

JOURNAL OF CELLULAR AND COMPARATIVE PHYSIOLOGY
Volume 49, Supplement 1, May 1957

SYMPOSIUM
ON
BIOCOLLOIDS

PUBLISHED BY
THE WISTAR INSTITUTE OF ANATOMY AND BIOLOGY
PHILADELPHIA
1957

SYMPOSIUM
ON
BIOCOLLOIDS

GIVEN AT
RESEARCH CONFERENCE FOR BIOLOGY AND
MEDICINE OF THE ATOMIC ENERGY
COMMISSION

sponsored by
THE BIOLOGY DIVISION
OAK RIDGE NATIONAL LABORATORY

Gatlinburg, Tennessee
April 12 - 14, 1956

OAK RIDGE NATIONAL LABORATORY

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INTRODUCTION

The step from enzyme and protein structure (1955 Symposium) to development of aggregates and biocolloids was discussed extensively at the 1956 Symposium held by the Biology Division, Oak Ridge National Laboratory, and the Division of Biology and Medicine, United States Atomic Energy Commission. This step is complicated and interpretation is difficult on the basis of our present knowledge; therefore it seemed to us to be a very worthwhile subject for discussion. In an evening session, the implications of fitting the aggregates and colloids into a biologically functional pattern were discussed. It will be obvious from this monograph that this is a field that demonstrates cooperation among biologists, chemists, and physicists. It will also be obvious that this field is still in the early stages of exploration and development.

The symposium also marked the first event in the series of thirteen International Developmental Biology Conferences, held in 1956, under the sponsorship of the Biology Council of the National Academy of Sciences-National Research Council, which was organized under the chairmanship of Dr. Paul Weiss of the Rockefeller Institute.

Doctor Norman G. Anderson, of this Division, had a major part in the arrangement of the program. His consultant services to the editor are gratefully acknowledged.

Previous symposia in this series are:

- 1948 — Radiation Genetics
- 1949 — Radiation Microbiology and Biochemistry
- 1950 — Biochemistry of Nucleic Acids
- 1951 — Physiological Effects of Radiation at the Cellular Level
- 1952 — Some Aspects of Microbial Metabolism
- 1953 — Effects of Radiation and Other Deleterious Agents on Embryonic Development
- 1954 — Genetic Recombination
- 1955 — Structure of Enzymes and Proteins

ALEXANDER HOLLAENDER

PREFATORY REMARKS

JOHN T. EDSALL

*The Biological Laboratories, Harvard University,
Cambridge, Massachusetts*

We have an enormous subject before us. There is nothing in science more vast in its scope than the subject of biocolloids. If we wish, we could include within our domain the whole of biochemistry, for everything in the living cell is either a biocolloid itself or it interacts more or less strongly with certain biocolloids. Even in a system such as blood plasma, which I have been concerned with for a good many years — a relatively inert system, perhaps, from the biological point of view — water, salts, urea, and everything else are affected to a very significant degree by being in the presence of the plasma proteins. Some are bound by one protein, some by another; some are bound strongly, others little, but none of them is completely in the free state, except perhaps the cations of the alkali metals, and I am not even sure about those. But in actively metabolizing cells, which respire and wriggle and twist and twitch, these interactions are far greater and far more dramatic than in blood plasma.

Since we have only three days here, we cannot discuss everything, although the capacities of this audience for covering the ground are great. Our major concern can be taken as defined by the interests of those who have come here to speak and to discuss. The biocolloids that we shall discuss do not, of course, cover the whole possible range. I might pay my respects here to starch, glycogen, hyaluronic acid, and all that great and goodly company of biocolloids that are carbohydrates or closely related to carbohydrate. Among them all, it seems probable that cellulose will be discussed at this

meeting by Professor Frey-Wyssling. I have recently listened to his fascinating series of Prather lectures at Harvard, and I am looking forward to the discussion that he will give this afternoon.

The dominant feature of the discussions that we are going to have appears likely to center on proteins and nucleic acids, what they do to the molecules that surround them, and what the surrounding molecules do to them. Proteins and nucleic acids, of course, are enormously reactive. These are molecules that are studded with polar groups, groups carrying electric charges, and groups that are capable of forming hydrogen bonds either as donors or as acceptors. They form a complete contrast to such systems as those of nonpolar polymers dissolved in inert solvents, long molecules that can turn and twist into a vast number of different configurations, all of which have very nearly the same energy. Instead of that, if you are going to change the configuration of a protein or a nucleic acid molecule, work is gained or lost in almost any change of configuration that can occur—the work of moving charged groups in an electrostatic field, the work of forming or breaking hydrogen bonds, perhaps the work of separating two or more nonpolar chains of various residues from one another and letting water molecules penetrate into the space that is opened up in this fashion.

In active molecules of this sort, different molecular configurations cannot all be considered as energetically equivalent. Some must be strongly favored over others from the point of view of energy. This is, of course, not to say that the native biocolloid is in its most stable configuration. Far from it. Probably the truth is more nearly in the opposite direction, that a macromolecule that is built up by a living organism to do a particular job, to catalyze a particular reaction, to serve as an agent of contraction and relaxation, or even perhaps to serve merely as a sort of cross link holding the framework of the structure together, is likely to have been built into a rather unstable state. The native biocolloid is probably subject to internal tensions and ready to change

into something else upon only moderate provocation. Yet it maintains a certain stability, having a pretty definite architectural form when it is isolated from its biological surroundings and suitably protected against disturbances after the chemist has extracted it from its natural state.

In handling biocolloids, I would emphasize that we are beginning to deal, and we shall be dealing more and more, with very definite molecules, not with complex and ill-defined mixtures. Thanks to the work of Sanger and his associates, we can now write the structural formula of three species of insulin just as we can write the structural formula of acetone or benzene. The same is true, or practically true, for several of the corticotropins that have been worked on by C. H. Li in California, P. H. Bell and his associates at Stanford, and in the Armour Laboratories at Chicago. It seems very likely that this will also be true before long of beef pancreas ribonuclease, which is a far more complicated molecule than the insulins. Ribonuclease, like insulin, appears to be a definite molecule with a uniquely defined sequence of amino acid residues, for any given animal species. Preparations that appear as mixtures of two or more components generally are found to differ simply by the conversion of a single side chain amide to a carboxyl group, or by some other equally simple change, not by a more far-reaching structural alteration. So, I think we can consider that biocolloids, even when they are not completely pure, are highly specific chemical entities. Indeed, it is often true that one can prepare them only in the form of rather complex mixtures of fairly closely related molecules, such, for instance, as the γ -globulin of plasma, which is not a single molecule at all, but a very complex array of different molecules. Yet this mixture is composed of closely related molecules that do in a sense form a family. I think this is probably also true of most, if not all, nucleic acid preparations that have been obtained up to now, but since I am not a nucleic acid chemist, I will leave that for others to judge.

The methods for resolving these complex mixtures into their individual constituents are, however, growing in scope and variety and power all the time, and I think that increasingly we shall be able to sharpen the definition of the molecules, the purity of the preparations, and to deal more and more with specific things. This is not to say, of course, that these definite molecules are necessarily rigidly restricted to any definite configuration. The very essence of behavior of many of the most important biocolloids is that they can change their configurations and change them in response to stimulation that might, from the point of most chemists, seem to be relatively mild. The classical example is the behavior of the actomyosin system of muscle; we shall hear much more about that in the final sessions of this conference. But there are plenty of other examples. The addition of a molecule of oxygen, for example, to a molecule of hemoglobin is not merely a local event that occurs in the neighborhood of the iron atom. It sets up far-reaching repercussions in the hemoglobin macromolecule that appear to lead to changes in its configuration, even a long way from the site of the chemical action. This was suggested several years ago independently by Pauling and Wyman, from the character of the interactions between the heme groups in hemoglobin, associated with the uptake of oxygen.

There is evidence of this in the difference in crystalline structure between reduced hemoglobin and oxyhemoglobin, from the changes in dielectric constant on oxygenation, recently reported by S. Takashima, and from the energy and entropy changes that occur in the successive steps of oxygenation. There are other lines of evidence, too. This is not, of course, just one isolated example. Similar changes of configuration in enzymes probably occur in some, perhaps most, of the reactions that they catalyze. They may, indeed, occur as a regular feature of enzyme-substrate interaction. This proposal is certainly not yet established, but it is definitely something to be considered.

At any rate, we are dealing with systems of molecules that interact with one another and with their surroundings to an extraordinary degree, to a degree that is strong and specific in a way that is not true in general of smaller molecules. One of the functions of this conference certainly is to try to trace out some aspects of that complex series of interactions.

PHASE SEPARATION IN POLYELECTROLYTE SOLUTIONS. THEORY OF COMPLEX COACERVATION

J. T. G. OVERBEEK¹

Falk Plaut Lecturer in Chemistry, Columbia University, New York

AND

M. J. VOORN

Laboratory of Physical Chemistry, University of Leyden

FIVE FIGURES

INTRODUCTION

It occurs frequently that solutions (even dilute solutions) of two polymers in the same solvent are immiscible or nearly so. Good examples are rubber and polystyrene dissolved in benzene or methylcellulose and polyvinylalcohol dissolved in water. Dobry and Boyer-Kawenoki ('47) have described a large number of cases of this "incompatibility," where each of the two phases formed is practically a pure solution of one of the two polymers. This phenomenon is explained by a negative heat of mixing of the two polymers, which means that heat has to be supplied for mixing. Even a small negative heat of mixing expressed per gram of polymer becomes a large effect if expressed per mole and can easily overcome the relatively small gain in entropy that may be seen as the "driving force" for mixing.

The phase separation in colloids studied by Bungenberg de Jong ('49a, b) and coworkers is of a fundamentally different type. Mixing of two aqueous solutions of gum arabic and gelatin at a suitable pH produces phase separation but in this type one phase contains most of the two polymers

¹ Permanent address: van't Hoff Laboratory, University of Utrecht.

combined with a modest amount of solvent, whereas the other phase is a dilute, often very dilute, solution of one or both the polymers. This type of phase separation occurs when the two polymers carry opposite charges. Bungenberg de Jong and Kruyt ('29) introduced the term "coacervation" and more specifically "complex coacervation" for this phenomenon. The Latin word *acervus* being heap, coacervation means literally coming together in a heap (of polymer particles), the prefix complex indicates that coacervation is brought about by a kind of complex formation between oppositely charged particles.

PHENOMENOLOGY OF COMPLEX COACERVATION

Complex coacervation has a number of characteristic features. Since naturally occurring polyelectrolytes often are polyampholytes, their charge depends on the *pH* of the solution. Complex coacervation is consequently sensitive to the *pH*. With gum arabic, a carbohydrate carrying carboxyl groups and gelatin, a protein carrying both carboxyl and amino groups, complex coacervation is possible only below *pH* 4.8, the isoelectric point of gelatin and above *pH* 2. Below *pH* 2, the dissociation of the carboxyl groups of the gum arabic is nearly suppressed.

Addition of neutral salts decreases the tendency to coacervation. In the presence of an excess of ions the charged groups on the polyelectrolyte are screened by the formation of dense ionic atmospheres around each charge, thus diminishing their interactions and consequently the mutual attraction between the polycations and the polyanions. It is obvious that this effect will be stronger the higher the valency of the added ions and that the suppression of coacervation by salts therefore should follow a Schulze-Hardy rule both with respect to the cations and to the anions

$$4-1 > 3-1 > 2-1 > 1-1$$

$$\text{and } 1-4 > 1-3 > 1-2 > 1-1.$$

Mention might be made here of the similarity in behavior of a complex coacervate and a globulin, both being "insoluble" in water but soluble in acids, bases, or salt solutions. The behavior of both might be subject to the same explanation.

The definitely liquid character of the complex coacervates was originally explained (Bungenberg de Jong, '49a, b; Bungenberg de Jong and Kruyt, '29) as a consequence of a strong hydration of the polymer particles. The electrostatic attraction that brings the particles together was supposed to be unable to press the water of hydration away, and the remaining hydration presumably allowed the coacervate to flow as a Newtonian liquid. This explanation was corroborated by the ability of alcohol, acetone, and similar "dehydrating agents" to promote coacervation, to make the coacervate more concentrated, and to counteract the influence of salts or of an unsuitable pH .

Bungenberg de Jong ('49a, b) gathered a large amount of data on complex coacervation. He varied the polyelectrolyte components and the salts, and sometimes used only one polyelectrolyte combined with oppositely charged polyvalent small inorganic ions (e.g., gum arabic and Th^{4+} salts) or replaced the polyelectrolyte by micelle-forming detergents. He pointed out that a far-reaching parallelism exists between complex coacervates and the behavior of biological membranes. Both swell or shrink under the influence of salts. Antagonism between Ca^{++} and Na^+ with Ca^{++} as shrinking and Na^+ as the swelling agent may be compared to the possibility of forming a complex coacervate between a negative polyelectrolyte ion and positive Ca^{++} or other polyvalent ions, the coacervation being suppressed by the addition of a sufficient amount of salts with ions of lower valency.

On account of the possible interest of complex coacervation to biology and because it is an interesting phenomenon per se, it seemed important that we look for a more quantitative theory.

GENERAL THEORETICAL CONSIDERATIONS

The problem is how to estimate the electrostatic attraction both with and without added salts, and how to find a suitable formulation for this rather mystical hydration.

Hydration or entropy. We approached the problem of hydration by considering phase separations for noncharged polymers. If a "bad solvent" is added to a solution of a nonpolar polymer, e.g., alcohol to a solution of polystyrene in benzene, a separation into two liquid phases occurs, one containing a high and the other a low concentration of the polymer. The solvent content of the concentrated phase has been explained as an entropy effect. With increase of the solvent content, the number of possible arrangements of this phase increases tremendously, with a corresponding increase in entropy; this is more important than the simultaneous (small) loss in energy of mixing. Quantitative treatments of this entropy of mixing effect have been given by Flory ('41, '42, '44) and Huggins ('42a, b). Tompa ('49) and Bamford and Tompa ('50) have developed the theory for phase separation in multicomponent systems. The same approach can be used for polyelectrolyte systems.

Electrostatic attraction. The electrostatic attraction seems to be difficult to evaluate for two polyelectrolyte coils. However, the coacervate is after all a rather concentrated solution and instead of containing clearly separated individual coils, it will be just a mass of intertwined coil molecules with the small ions distributed between them. The interaction between individual charged spots and ions will be much more pronounced than the interaction of complete polyelectrolyte molecules. The electrical free energy of the whole coacervate, therefore, may suitably be approximated in the same way as the electrical free energy of an electrolyte solution, according to the Debye-Hückel theory (Debye and Hückel, '23).

We thus shall calculate the electrical free energy by assuming that the influence of the polyelectrolytes on it is the same as that of an equivalent number of monovalent

ions. In the coacervate this picture will not be too unreasonable. It may be rather unsatisfactory in very dilute equilibrium liquids, but then the contribution of the poly-electrolytes to the electrical free energy is small anyway and an error in the calculation will not be very important.

If we use these approaches for the entropy of mixing and the electric free energy and neglect the possible contribution of short-range (van der Waals) forces to the energy of mixing, it is not difficult to formulate a quantitative theory of complex coacervation.

QUANTITATIVE FORMULATION OF THE ENERGY

Consider a volume V of solution containing n_i molecules (or ions) of species i , the molecular partial volume of each species being $r_i v$, where v is the molecular partial volume of the solvent. The charge per ion will be given by $z_i e$, e being the elementary charge. In order to keep the equations as simple as possible, we will assume that the molecular volumes of all small ions are also equal to v , and that of the polyions to rv , the charge of the polyions being either $+ze$ or $-ze$. For convenience, the ratio $z_i/r_i = \sigma_i$ will be introduced and called charge density. For the solvent it will be zero, for the small ions 1, and for the polyions a number between 0 and 1.

According to the theory of Debye and Hückel ('23), the electrical free energy of the solution in the volume V is given as follows:

$$F_{el} = - \frac{e^2}{3\epsilon} \kappa N_z \quad (1)$$

with

$$\kappa^2 = \frac{4\pi e^2 N_z}{\epsilon k T V} \quad (2)$$

where ϵ is the dielectric constant of the solvent,

e the elementary charge,

$N_z = \sum n_i |z_i|$ = the total number of elementary charges (+ and -),

k = Boltzmann's constant,

T = absolute temperature.

Introducing volume fractions $\phi_i = \frac{n_i r_i v}{V}$ and the ratio $N = V/v$ of the total volume V to the elementary volume v , the equation for F_{el} can be written:

$$\frac{F_{el}}{NkT} = -\alpha [\sum_i \sigma_i \phi_i]^{3/2} \quad (3)$$

with

$$\alpha = \left(\frac{e^2}{\epsilon kT} \right)^{3/2} \cdot \frac{2}{3} \sqrt{\frac{\pi}{v}} \quad (4)$$

For water as a solvent and room temperature, α is about 3.5.

Typical features of F_{el} to which we shall return presently are:

(1) It is proportional to the 3/2 power of the concentration of the total charge; (2) it is independent of the distribution of the charge between polyelectrolyte and small ions; and (3) it is inversely proportional to the 3/2 power of the dielectric constant.

These three features will be preserved in other more refined models although presumably in a less simple form. The electrical free energy will be:

(1) Proportional to a power higher than the first power of the concentration of charges, (2) not very dependent on the distribution of charges, and (3) increasing strongly with decreasing dielectric constant.

Turning now to the entropy of mixing, we shall use the Flory-Huggins expression (Flory, '41, '42, '44; Huggins, '42a, b),

$$TS_{mix} = -kT \sum_i n_i \ln \phi_i. \quad (5)$$

Dividing by NkT , we find

$$\frac{TS_{mix}}{NkT} = -\sum_i \frac{\phi_i}{r_i} \ln \phi_i. \quad (6)$$

The most characteristic feature of the entropy of mixing is the relatively small contribution of the polyions. This is caused by the factor $1/r$. This feature will also be preserved in more refined models.

The complete expression for the free energy of a polyelectrolyte solution that will be used in further derivations is thus

$$\frac{F}{NkT} = -\alpha [\Sigma_i \phi_i]^{3/2} + \Sigma_i \frac{\phi_i}{r_i} \ln \phi_i. \quad (7)$$

TWO-COMPONENT SYSTEMS

As a rule, complex coacervating systems are multicomponent systems containing water, two polymers, one or more salts, and possibly nonionic substances such as alcohol.

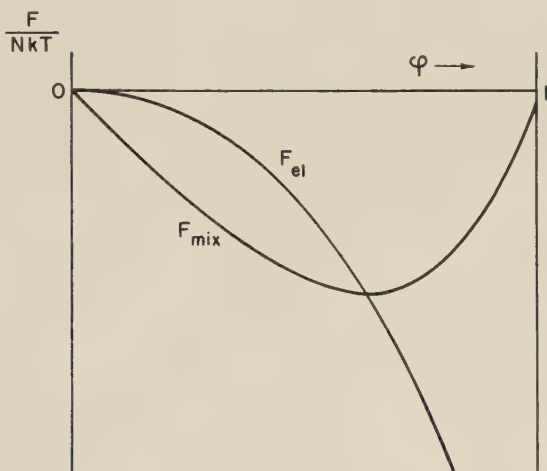


Fig. 1 Electrical part (F_{el}) and entropy of mixing parts ($F_{mix} = -TS_{mix}$) of free energy of mixing plotted against volume fraction of polymer PQ.

The most simple system is evidently a two-component system consisting of water and a polymer salt PQ composed of z -valent polycations P^{z+} and z -valent polyanions Q^{z-} , both with the same molecular volume rv .

In this way a system is obtained with only one concentration variable, for which we take

$$\phi = \phi_P + \phi_Q.$$

Equation (7) can then be written

$$\frac{F}{NkT} = -\alpha (\sigma\phi)^{3/2} + (1-\phi) \ln (1-\phi) + \frac{\phi}{r} \ln \frac{\phi}{2}. \quad (8)$$

Figure 1 shows the electrical part and the entropy of mixing part of F/NkT plotted separately against ϕ . As is normal

in this kind of representation, the curvature in the entropy of mixing curve is positive. The electrical free energy has a negative curvature. The combined curve, representing the total free energy can have either a positive curvature for all values of ϕ or a negative curvature in the middle part and positive curvatures at the two ends as shown in figure 2. In the latter, phase separation will occur in the system, the composition of the two coexistent phases is given by the ϕ values of the tangent points of the bitangent to the curve.

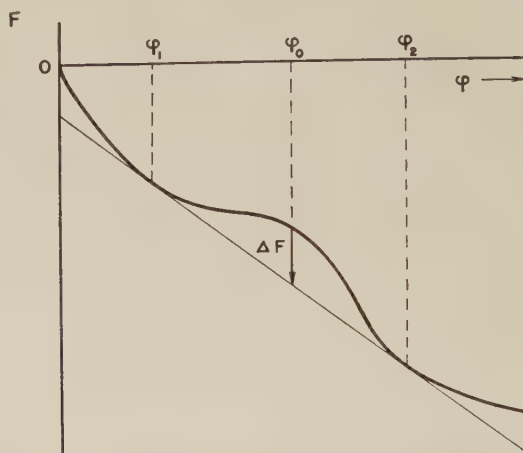


Fig. 2 Free energy versus volume fraction in a system showing demixing. ΔF is the decrease in free energy when a mixture of composition ϕ_0 is split into two phases with compositions ϕ_1 and ϕ_2 .

For any mixture between the compositions ϕ_1 and ϕ_2 the free energy of the two phase system, as given by a point on the bitangent, is lower than that of the one-phase system represented by the corresponding point on the curve.

Obviously, a curve of the type represented in figure 2 has two inflection points. In a critical curve, representing a situation just on the verge of unmixing, these two inflection points have merged. This leads to the mathematical condition for the critical point:

$$\frac{d^2 F}{d\phi^2} = 0 \quad \text{and} \quad \frac{d^3 F}{d\phi^3} = 0. \quad (9)$$

Application of these conditions as expressed by equation (9) to equation (8) shows that the critical condition is obtained with good approximation if

$$\sigma^3 r \approx 0.5. \quad (10)$$

This shows that for complex coacervation to occur, either the charge density σ or the molecular weight r or both have to be sufficiently large.

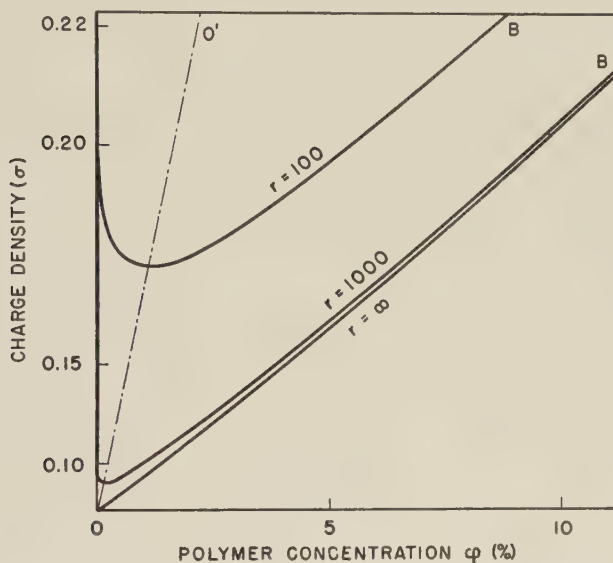


Fig. 3 Influence of charge density σ and chain length r on phase separation. The figure has been constructed from equation (8) with $\alpha = 3.655$. The drawn lines represent the composition of the two phases, the dotted line $00'$ is the locus of the critical points.

Figure 3 shows lines representing the two coexistent phases for combinations of σ and r . The critical points are found on the line $00'$, the right-hand branches of the curves represent the coacervates, the left-hand branches the equilibrium liquids. It is gratifying that for reasonable molecular weights ($r=1000$ would correspond to $M \sim 20,000$), the charge density should exceed 0.08 (i.e., the equivalent weight should be below about 250) and that the concentrations of the co-

acervates are of the order of a few per cent, which is in reasonable agreement with actual experimental figures.

Calculations on these and on the more complicated systems are given in greater detail in the thesis of the second author (Voorn, '56). The equilibrium concentrations are derived from the condition that the chemical potential for each individual species has to be the same in the two phases. The chemical potentials are obtained by differentiation of the free energy with respect to the concentrations.

THREE-COMPONENT SYSTEMS

More important conclusions can be drawn from the study of symmetrical three-component systems, i.e., systems that can be considered to consist of the solvent, a polymer-polymer salt PQ, and a simple mono-monovalent salt KA.

A graphical representation of the free energy against the concentrations now demands a three-dimensional figure with ϕ_{polymer} and ϕ_{salt} along two axes and F along the third (see fig. 4). Increasing the salt content will bring the composition of the two coexistent phases closer together. This can be explained qualitatively in the following way. The decrease in free energy in phase separation is due to the more than proportional increase in the electrical interaction ($\phi^{3/2}$) in accumulating the polymer in a concentrated phase or, in other words, by the fact that in concentrating the polymers the radius of the ionic atmosphere is decreased, which increases the electrical interaction. Now if salt is present, it will be distributed nearly equally over the two phases because any markedly uneven distribution would give rise to an intolerably high decrease in the entropy of mixing. Consequently, there is already a considerable and nearly equal amount of ions in the two phases and accumulation of the polymer in one of the two phases will only change the radius of the ionic atmospheres to a smaller extent, thus making the gain in free energy smaller than in the absence of salt. The compositions of the two phases, therefore, come closer

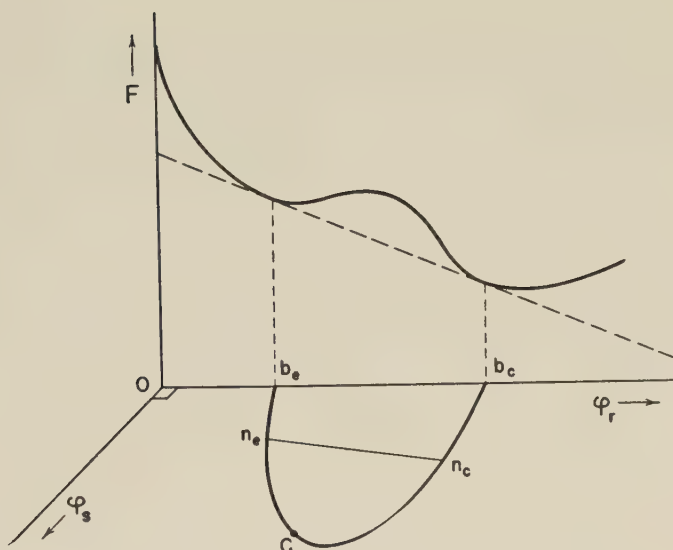


Fig. 4 Free energy versus concentrations in a three-component system. ϕ_r is the volume fraction of the polymer PQ and ϕ_s that of the salt KA. The line $b_e C b_c$ is the projection of tangent points of the bitangents to the F plane; $n_e n_c$ is a node line, connecting coexistent phases.

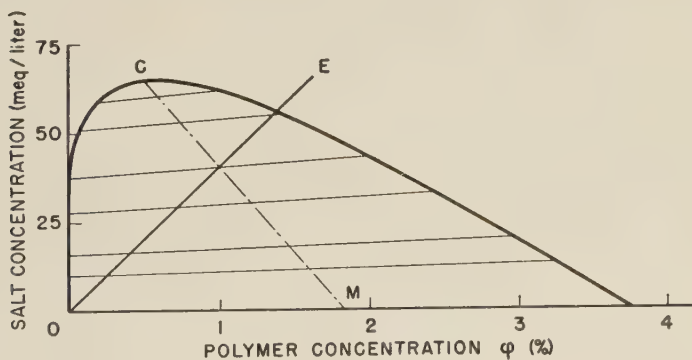


Fig. 5 Phase diagram for complex coacervation in the system solvent, polymer PQ and univalent salt KA. The figure has been constructed for $r = 1000$, $\sigma = 0.15$, and $a = 3.655$. Polymer concentrations are expressed in volume per cent. In the conversion of the salt concentration to milliequivalents per liter it has been assumed that the molar volume of the ions is 18 cm^3 , the same as that of water.

together with increasing salt content, and finally reach a critical point, beyond which no phase separation occurs. The curve $b_e n_e C n_e b_e$ in figure 4 represents the projection onto the $\phi_r - \phi_s$ plane of the tangent points of the bitangents of the F plane. C is the critical point. Figure 5 gives an actual example of the calculated equilibrium figure for the case $r = 1000$, $c = 0.15$, and $\alpha = 3.655$.

The conversion of volume fractions ϕ to concentrations in milliequivalents per liter is effected by assuming the molar volume of the solvent and that of the monovalent ions to be 18 cm^3 . The line OE corresponds to equivalent amounts of polymer and salt. If the coacervating system is prepared from the two polyelectrolytes each neutralized with monovalent ions (i.e., from PA and QK) with the possible addition of monovalent salt KA, the initial systems are all on or to the left of the line OE.

The line MC is the locus of the middles of the node lines. It is very close to a straight line. Coacervating systems of this type follow, therefore, the law of the rectilinear diameter first found by Cailletet and Mathias (1886) in gas liquid equilibria.

The node lines do not run parallel to the polymer axis but they indicate a slight excess of salt in the coacervate. This is again in agreement with qualitative expectation because the coacervate contains more charges per unit volume and consequently has stronger interaction among the charges, thus lowering the free energy. The effect however can only be small on account of the large entropy contribution of the small ions.

This consequence of the theory had never been tested. We did some experiments on complex coacervates of the K salt of gum arabic and gelatin chloride and found indeed that the coacervate contained about 10% more KCl than the equilibrium liquid, the concentrations being expressed per unit volume of the phases (see table 1).

FOUR-COMPONENT SYSTEMS

Dropping the condition of symmetry and allowing systems in which the amounts of polycation and polyanion are no longer equivalent, we arrive at four-component systems. Ex-

perimentally, the choice of components will probably be water, KA, QK, and PA, but other possibilities exist to describe the same systems. A choice in which the four-component systems can be more easily derived from the three-component ones is water, PQ, KA, and PA (or QK, depending on which of the polymers is in excess).

As compared to the symmetric systems, a new variable appears, i.e., the potential difference between the two phases. If the polycations are in excess, the potential of the coacervate is positive with respect to the equilibrium solution.

TABLE 1

Distribution of KCl in a coacervated system of gelatin-chloride and potassium arabinat

40°C.; pH, 3.75; polymer concn. in coacervate, 14% by wt., in equil. liquid, 0.5% by wt.									
EXPT. NO. →	1a	1b	2a	2b	3a	3b	4a	4b	MEAN
<i>concn. of KCl in meq/liter</i>									
In coacervate (C_c)	9.2	8.7	9.3	9.3	8.8	8.9	8.7	9.0	9.0
In equil. liquid (C_p)	8.24	8.29	8.25	8.25	8.34	8.33	8.32	8.29	8.29
C_c/C_p	1.08		1.12		1.06		1.06		1.08

Although this potential difference does not occur explicitly in the expression for the free energy of the phases [eq. (7)], because the phases are considered to be electroneutral, it cannot be neglected when equilibrium conditions are derived by use of equality of the electrochemical potentials of each species in the two phases.

If PA is in excess, it will accumulate like any other salt in the coacervate. However, because of the large number of small ions A, this is unfavorable from an entropy point of view and therefore the coacervate will be more symmetric than the original mixture, whereas the equilibrium liquid will be more asymmetric, containing a good many P^+ ions but very few Q^- ions. Table 2 shows the calculated distribution for the polymer-polymer salt PQ, the excess species

PA and the electrolyte KA between the two phases for a representative case. It is very striking how close the distribution coefficient of KA remains to unity, how extreme that of PQ may be, and how that for PA is intermediate never exceeding 10.

If the asymmetry is too large, coacervation is completely prevented. One might say that the excess polymer PA acts as added electrolyte to the polymer-polymer salt PQ.

Although a mixture of a single polyelectrolyte PA with water is not expected to show coacervation, and indeed does

TABLE 2

Calculated distribution coefficients of salt KA, polyelectrolyte PA, and polymer-polymer salt PQ in nearly symmetric systems (very small excess of PA)

$$r = 1000, \sigma = 0.15, a = 3.655$$

SALT CONCN. IN EQUILIBRIUM LIQUID	DISTRIBUTION COEFFICIENT OF:		
	KA	PA	PQ
<i>meq/liter</i>			
16.0	1.26	9.76	6130
21.9	1.20	2.09	402
51.3	1.10	1.18	14
56.3	1.07	1.12	6
59	1.05	1.08	4
62.7 ^a	1.025	1.04	2
64.35 ^a	1.00 ^a	1.00 ^a	1.00 ^a

^a Critical point.

not show it, exchange of the monovalent counter ions for divalent ones (lowering of entropy contributions) or addition of such substances as alcohol or acetone that decrease the dielectric constant (increase of the electrical interaction) may bring the system to coacervation. I. Michaeli (private communication) has found this behavior in the sodium and calcium salts of polymethacrylic acid. Bungenberg de Jong ('49a, pp. 384ff) has described a number of cases of so-called "auto-complex-coacervation" in systems of one polyelectrolyte with bi- or polyvalent gegenions.

RELATIONS TO BIOLOGY

Complex coacervation is believed to be important for biology in several respects.

The parallel with the behavior of biological membranes and with the solubility of globulins has already been mentioned in the Phenomenology section.

Interaction of two proteins or of protein and nucleic acid in nucleoproteins may very often contain, in addition to a specific part, an important interaction of the complex coacervate type, and consequently may be sensitive to salts and pH changes.

Also, in a preparative sense, complex coacervation may find its uses in treatment of mixtures and possibly in processes of fractionation.

CONCLUSION

The main object of this paper is to draw attention to the very general nature of complex coacervation, to show that it can be explained theoretically by simple general principles, and in particular to show that the difference in behavior between large molecules and small ones is mainly due to entropy effects, whereas the energies of interaction, when expressed per unit weight, are of the same order for large and for small molecules.

SUMMARY

A brief phenomenological description is given of complex coacervation, the phase separation occurring in polyelectrolyte systems due to interaction of the electric charges of the polyions. Complex coacervation is treated theoretically as a competition between electrical attraction tending to accumulate the charged particles and entropy which tends to disperse them. The treatment is put on a quantitative basis by use of the Debye-Hückel equations for the electrical interaction and the Flory-Huggins theory for the entropy. The properties of free energy versus composition curves or planes are used to treat two-phase equilibria.

Systems of different complexity of composition are treated briefly, the most complicated one consisting of water, two polymers of opposite charge, an electrolyte and a nonionized substance of low dielectric constant.

It is shown experimentally and theoretically that not only the polymers but also the low molecular weight salt is accumulated in the coacervate.

Possibilities of application in systems of biological interest are mentioned.

GENERAL DISCUSSION

SCHERAGA ²: In your free-energy diagram you have a range of compositions in which the system of two polymers and one solvent can minimize its free energy by splitting into two phases. Doesn't this imply that the ratio of one polymer to the other is fixed?

OVERBEEK: Here I assumed that the two polymers were present in equivalent amounts, but it is not necessary to assume this. One can use the same treatment for nonequivalent amounts of polymer, but then one has to add one other axis. Nonequivalent amounts of polymer can occur only in systems containing three or more components.

MEL ³: Do you consider the main difference between the coacervation complex here and, say, an ordinary crystal process where you have an included solvent to be the amount of solvent actually trapped there?

OVERBEEK: Not only that. The complex coacervate is really liquid. It may be a viscous liquid; the viscosity may be 50 or 100 times that of water. But there is no X-ray diffraction pattern. There is no ordered arrangement of the water molecules, nor is the water content of the coacervate a constant.

SZENT-GYÖRGYI ⁴: Would not the behavior be modified considerably if the two charges of the macromolecular chromosomes were the bivalent kind?

² Harold A. Scheraga, Cornell University.

³ H. C. Mel, University of California.

⁴ Albert Szent-Györgyi, Marine Biological Laboratory, Woods Hole.

OVERBEEK: I am sure that has an influence. You may remember from the Introduction that Bungenberg de Jong has also studied complex coacervates formed from only one polyelectrolyte. If the same approach is applied to a potassium salt or a sodium salt of a polymer, no phase separation is found, but as soon as the calcium salt is taken, coacervation may occur because the contribution of the bivalent calcium ion to the entropy is less important than that of an equivalent amount of monovalent ions.

It is easy to predict that if on the polymer elementary charges are present in pairs, so close together that they should be considered as bivalent ions, this should be a factor in favor of coacervation.

SZENT-GYÖRGYI: This is because many of our biological systems do form coacervates when they should not.

OVERBEEK: When ATP is present quite a number of charges are concentrated and that will enhance the coacervation effect.

RANDALL⁵: A solution of gelatin consists of a system of random coils according to the evidence of light scattering and there are charges of both signs on any one molecule. Your analysis, so far as I understand it, completely avoids the shape factor in the configuration of the molecule. Is that so?

In solutions of such molecules as collagen the process of precipitation may occur. Does your analysis in any way cover the case where the molecule has both signs of charge on it and, secondly, does it cover the case that Flory has dealt with in a rather general way, in two recent papers, of the molecules coming together to form fibrils?

Of course, in collagen, which is a more complex system than those dealt with by Dr. Overbeek, molecules come together in what amounts to a process of crystallization; and all the structures that Dr. Schmitt and his colleagues and others have shown to exist in these precipitates take time to form.

⁵ J. T. Randall, University of London King's College.

I will be interested to know if your analysis throws any light on these problems.

OVERBEEK: I would say that the presence of charges of two signs on one molecule can be easily included in the treatment. It is just a question of considering the charge density factor in a little more general way. So far, in our calculation on gelatin, we have considered the charge density as representing the net charge of the gelatin molecule. But we might, of course, also say there is a larger positive charge and a smaller negative charge and they should be included independently. That would not make the treatment more complicated. It would favor coacervation.

Specific forms and the fitting of two molecules more or less onto each other are certainly not included and I should like to see other people work on this aspect.

EDSALL⁶: I presume there are systems that are still outside the critical point where a phase separation does not occur, and by the use of methods such as light scattering one would detect changes caused by the interactions that would vary somewhat with the ionic strength or the dielectric constant and other factors that might be picked up.

OVERBEEK: I don't think there have been any serious experiments in this field, but it would be interesting to go close to the critical point and see whether interactions could be discerned.

EDSALL: I have a particular case in mind, a system that was investigated by light scattering in our laboratory about 10 years ago by Peter Morrison, a system composed of γ -globulin at a pH of about 5.6 and that was negatively charged—and most of the γ -globulin is positively charged—where at low ionic strength there was a lot of precipitation that, in the absence of either component, was perfectly soluble. But even apart from this, where there was no actual precipitation there was a marked increase in the turbidity of the system that could be followed very nicely and varied in a

⁶J. T. Edsall, Harvard University.

striking fashion with a variation in the ionic strength of the system. Here, of course, there are two molecules that are more or less globular proteins. They do not correspond to the particular model that you have assumed, but I think that qualitatively there must be a general resemblance.

OVERBEEK: May I make just a short remark here? One should be careful in considering the turbidity that is obtained in this way as an equilibrium property. It may be that very small droplets of the complex coacervate are formed, and if the situation is such that these droplets carry, e.g., a negative charge, if the coacervate is unsymmetrical, then by the repulsion between the negative charges the formation of one continuous phase may be prevented. We have seen such cases very often.

ANDERSON⁷: I was interested in the comment about the interaction between proteins, for example, in the nucleus and the deoxyribonucleic acid. As I understand it, coacervates are equilibrium systems. One would therefore not be able to isolate a little coacervate globule, whereas a nucleus can be taken out of the cell and will exist without dissolving in solutions of ordinary ionic strength such as probably obtain in cells. But some idea of the lability of the bond between the protein and nucleic acid can be gotten from the fact that if one puts in a large molecule with a higher charge density than either the protein present or the nucleic acid present, such as heparin or protamine, one can displace out the substance of the same charge.

OVERBEEK: I would just like to remark that it would be rather difficult to understand, if the nuclei were just a complex coacervate, why the two nuclei do not melt together. On the other hand, the equilibrium concentration of the coacervate may be so low that even a single nucleus in a comparatively large amount of liquid will not dissolve readily. The distribution ratio can be very extreme.

⁷ N. G. Anderson, Biology Division, Oak Ridge National Laboratory.

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THE PHYSICAL CHEMISTRY OF DEOXYRIBONUCLEIC ACIDS

PAUL DOTY

*Department of Chemistry, Harvard University,
Cambridge, Massachusetts*

FIVE FIGURES

During the last few years deoxyribonucleic acids (DNA) have become one of the principal focal points in science. This is the result of increasingly convincing demonstrations that they are the chief carriers of genetic information and therefore hold within their structures the blueprints of all the other cellular constituents. The structure and function of DNA is proving sufficiently complex to sustain for years to come the best efforts of the practitioners of many scientific disciplines. The purpose of this lecture is to report the current state of developments from one sector, that in which the techniques and concepts commonly classed as physical-chemical have been applied to the investigation of the structure and properties of DNA. From the occasional glimpses that these studies provide, one can put together a fairly consistent view of what is regarded by many as Nature's highest achievement in molecular architecture.

By way of introduction the isolation, chemical composition, and chemical linkages are briefly considered. DNA occurs in conjugation with basic proteins, histones or protamines, in the nuclei of cells and in a less well-defined state in lower organisms such as bacteria, phages, and animal viruses. The demonstration of progress in the isolation of DNA without the breakage of either primary or secondary bonds depends on the

evolution of methods of characterization that permit the detection of the type of damage that it is desired to avoid. With the methods of characterization now at hand it appears that very great progress has been made over the last decade in the empirical refinement of preparative procedures and in the working out of new ones. To Chargaff's review ('55) of these procedures there should be added the one developed by Simmons ('55) (making use of sodium xylene sulfonate to dissociate and remove protein), by which many of the samples used in the work discussed here were prepared. It has been found that the tissue or other source of DNA must be frozen in dry ice immediately upon removal from the animal if autolytic degradation is to be completely eliminated. Isolation procedures must insure the DNA against any enzymic attack or other degradative conditions such as exposure to extremes of pH, temperature, shear stresses and excessive drying. Traces of protamines and histones as well as polyvalent cations must be eliminated to reduce or prevent aggregation. As noted below it is also essential that DNA be prevented from dissolving in water at high dilution since a cation concentration of approximately $10^{-3} M$ is required to protect the native configuration at room temperatures and at neutral pH.

From a chemical point of view, DNA is a polymer of nucleotide units linked together with phosphodiester linkages. The phosphate group of the nucleotide is joined, through a five-membered sugar ring that is deoxyribose in every case examined, to one of four bases. The bases are adenine, cytosine, guanine, and thymine: in rare cases 5-methylcytosine replaces cytosine to some extent and in some phages DNA cytosine is replaced entirely by 5-hydroxymethylcytosine. Careful analytical work allowed Chargaff to conclude in 1950 that the composition of most DNA samples is such that adenine and thymine predominate or that guanine and cytosine predominate. Further work established that in most cases the mole ratios of adenine to thymine and guanine to cytosine were unity (Chargaff, '50, '55).

Convincing evidence is now available to show that the inter-nucleotide union consists of a 3',5'-phosphodiester linkage. The chemical skeleton of DNA can therefore be sketched as shown in figure 1.

THE CONFIGURATION OF DEOXYRIBONUCLEIC ACID

If DNA consisted simply of single molecular strands of nucleotides, rotation about many of the backbone bonds would cause it to exhibit properties similar to those characteristic of polyelectrolytes. About 1951 we began to realize that such was not the case. Streaming birefringence measurements made at different ionic strengths (Schwander and Signer, '51) showed little variation, indicating that the DNA was relatively rigid

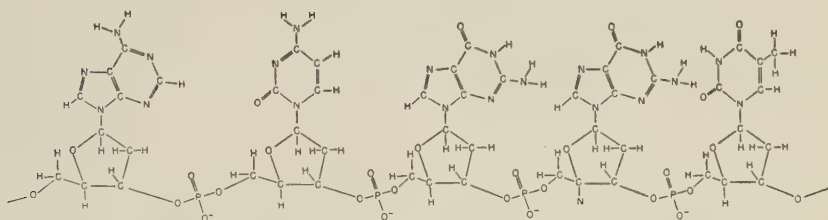


Fig. 1 The chemical structure of deoxyribonucleic acid. Adenine, guanine, cytosine, and thymine are the base groups that occur in unknown sequence.

and not easily deformable. Early light scattering measurements (Doty and Bunce, '52) indicated that a several-stranded structure existed. When the ionic strength dependence of the specific viscosity became clear (Conway and Butler, '54), it was evident that DNA did not expand appreciably as the ionic strength was decreased. Taken together these early observations clearly suggested that the configuration of DNA in solution was too rigid and too highly extended in space to have the randomly coiled configuration of a long polynucleotide chain.

For some time prior to 1953 excellent X-ray fiber diagrams of DNA had been obtained by Wilkins, Franklin, and their colleagues at King's College, London. When the general features of these became known, Watson and Crick ('53; Crick and

Watson, '54) were able to propose a novel structure based on detailed consideration of new data on bond angles and distances, together with an original idea of base pairing through hydrogen bonds. They argued that two polynucleotide chains could be hydrogen-bonded together in the form of a double

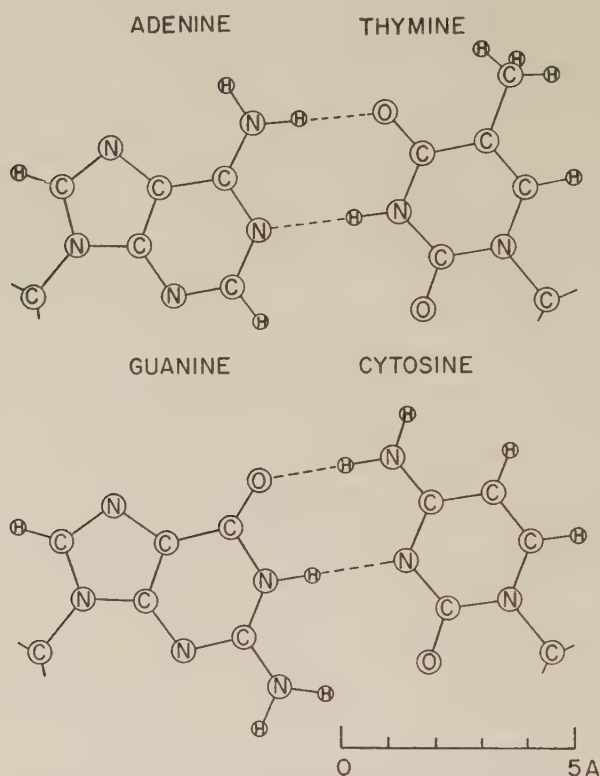


Fig. 2 Hydrogen bonding between base pairs in the Watson-Crick structure for DNA.

helix with the phosphate groups on the outside provided that the bases were perpendicular to the helix axis and that adenine bonded only with thymine and guanine only with cytosine. These two base pairs are shown in figure 2 where it is important to note that all the atoms shown should lie in the plane of the paper. The structure repeats every 34 Å, accounting for

the strong meridional reflection observed at that distance. There are ten nucleotide pairs per turn of the helix, that is, one every 3.4 Å, which accounts for the layer line spacing. The analytical findings of Chargaff, Wyatt, and others on the molar equivalents of adenine and thymine and of guanine and cytosine have in this structure a natural explanation. This structure received general confirmation from the X-ray investigations (Franklin and Gosling, '53; Wilkins *et al.*, '53). Later studies of the optical transform of the proposed structure show that the bases must lie somewhat closer to the center of the helix and the orientation of the phosphate groups must be altered if good agreement with the observed diffraction is to be achieved. Since DNA is highly crystalline it is clear that the structure derived from the X-ray diffraction does exist in at least half of the sample and there is no evidence that seriously suggests that the remainder is particularly different. The X-ray evidence alone, however, cannot rule out any structural feature proposed to be present in minor amounts.

The next question that arises is whether the Watson-Crick structure is preserved in solution and, if so, whether it accounts for all DNA present. The compatibility of this model with the early observations just summarized suggests that this structure may be present in solution and in the next section, where we undertake to summarize the behavior of DNA in solution, this question may be examined quantitatively.

THE PROPERTIES OF DEOXYRIBONUCLEIC ACID IN SOLUTION

In order to elucidate the characteristic features of isolated DNA molecules it is necessary to measure various physical properties of solutions that are so dilute that the molecules are for the most part independent of one another. This state of dilution is required for proper extrapolation of physical measurements to infinite dilution where the molecular properties, free of any interaction effects, can be unambiguously determined. For rather compact macromolecules like globular

proteins this concentration range in which meaningful measurements can be made extends as high as 5 or 10 g per deciliter. For typical polymers having molecular weights of several million, it extends only to approximately 0.5 g/dl. Experience shows that with DNA solutions it extends only about as high as 0.02 g/dl. This situation points out at once the special problems that pertain to the determination of the molecular properties of DNA in solution. It is necessary to use only those methods that remain sensitive at very high dilution. During the last 5 years in which intensive work has been carried on in the physical-chemical characterization of DNA the methods of light scattering, viscosity and sedimentation, after proper adaptation, have provided increasingly reliable data on solutions of sufficient dilution, whereas many of the other classical methods such as osmotic pressure and diffusion have not proved useful. Each of these three successful methods will be considered, the latest developments being emphasized.

Light scattering

The determination of the angular intensity distribution of light scattered from a macromolecular solution can generally be interpreted to give the molecular weight and size (radius of gyration) of the dissolved macromolecule. In addition, the net attraction or repulsion of the solute molecules can be estimated and, in favorable cases, some information about particle shape and polydispersity (i.e., the distribution of molecular weights) can be obtained.

The average molecular weight, size, and shape of DNA. The early investigations on light scattering were carried out on samples that can now be judged as having been slightly degraded or denatured, at least in some cases (Doty and Bunce, '52). But the first results on a Schwander and Signer preparation from calf thymus gave results that do fall within the range now accepted. In this particular case a molecular weight of 6.7 million and a radius of gyration of 2600 Å were obtained.

Measurements on a number of carefully prepared samples from this tissue (Reichmann *et al.*, '54; Rice and Doty, '57) indicate that the average molecular weight can be assigned as 8 ± 1.5 million and the radius of gyration as 2950 ± 300 Å. The ranges shown for these figures arise from both experimental errors and small but real variations among the samples.

When the molecular size is as large as that observed here, the reciprocal scattering envelope should reveal some information about the molecular shape. The downward deviations of the reciprocal scattering envelope [Kc/R_{θ} versus $\sin^2 \theta/2$] from the linear behavior found at low scattering angles seemed best interpreted (Doty and Bunce, '52; Rice and Doty, '57) as caused by the DNA molecules having highly extended, gently coiled configurations, comparable for example to those of the common earthworm. With this conclusion concerning the molecular shape, the radius of gyration can then be translated into the root-mean-square end-to-end length of the DNA molecule: this mean distance is 7200 ± 700 Å. This size refers to solutions containing 0.2 *M* NaCl, but it remains constant except for very low ionic strengths.

Samples of DNA prepared by Dr. N. S. Simmons from pneumococcus, chicken erythrocytes, and salmon sperm have molecular weights and sizes within the ranges given here for thymus DNA (N. S. Simmons, S. A. Rice, M. Litt, and P. Doty, unpublished results). These observations probably should not be generalized. Determinations by light scattering of molecular weight as high as 15 million have been reported for DNA from other sources (Brown *et al.*, '55) and there is some indication that DNA from T2 phage is substantially larger than that of thymus. When deciding whether new results should be accepted we must keep in mind that any degradation during the preparative procedure can give anomalously low values and, on the other hand, the incomplete removal of protein can lead to anomalously high values as a result of the protein binding DNA molecules together.

The molecular configuration of DNA in solution. The downward curvature in the reciprocal scattering envelope of DNA could arise from excessive polydispersity or from an unusually low degree of local curvature in the DNA molecule. For the present it will be assumed that the polydispersity is such that it does not cause curvature in the envelope. As a consequence of this assumption, the molecular weights obtained from light scattering correspond to the weight average values (M_w) and the root mean square end-to-end length to the z -average value (r_z). On this basis it is useful to interpret the latest angular intensity data in terms of the analysis proposed by Peterlin ('53) who became interested in this problem as a result of the first light-scattering data published on DNA.

By adopting the "worm-like" chain model of Kratky and Porod, Peterlin succeeded in relating the degree of downward curvature in the reciprocal envelope to the number and length of Porod units with which the molecule can be represented. The Porod unit, designated as q , is a "persistence length," defined as the integral of the average projections of chain elements of an infinitely long chain on its initial direction. (In terms of the more familiar Kuhn model composed of rodlets — statistical elements — connected by universal joints, the persistence length is equal to twice the length of the Kuhn elements.) Moreover, when the number of Porod units has been assigned for a sample of known molecular weight (M_z) the value of q can be obtained from r_z and this in turn leads to the value of the mass per unit length, M/L , through the relations

$$x = L/q = (M/q)/(M/L) \text{ and } q = r_z/(2x)^{1/2} \quad (1)$$

where x is the number of Porod units per molecule.

To carry this procedure through we note that the reciprocal scattering envelopes for three recent scattering experiments on thymus DNA (Rice and Doty, '57) can be closely approximated by the curve calculated from Peterlin's work for $x = 100$. The persistence length is then found to be 500 Å, and this yields a value of 230 molecular weight units per Å for M/L . This compares favorably with the value of 200 required

by the Watson-Crick model and offers support to the contention that the observed curvature in the reciprocal envelopes does indeed arise from homologous polymer chains of high extension and not from an extremely broad molecular weight distribution.

The exceptionally low degree of coiling in DNA indicated by the length of 500 Å for the Porod unit is better appreciated when some comparisons are made with cellulose trinitrate, which is generally considered to be highly extended, and polystyrene, a typical highly coiled synthetic polymer. This is done in table 1, where in addition to the persistence length there is shown the end-to-end length and the molecular weight

TABLE 1
Comparison of chain configurations of DNA and other polymers

SUBSTANCE	PERSISTENCE LENGTH (q)	MOLECULAR WEIGHT OF KUHN ELEMENT	WHEN $r = L/4$:	
			r	mol. wt.
DNA	500 Å	200,000	5200 Å	6,300,000
Cellulose trinitrate	117	13,500	950	200,000
Polystyrene	9	740	164	27,000

at which r becomes equal to one-fourth the contour length, L . At molecular weights lower than this, the departures from Gaussian chain statistics become increasingly pronounced. But investigations of cellulose nitrate (Benoit *et al.*, '54) down to molecular weights where r is no more than one-fourth the contour length have revealed no anomalies in the molecular weight dependence of intrinsic viscosity and sedimentation rate, although in this limit the relation of the end-to-end length and the molecular weight has deviated by a factor of 2 from the asymptotic value at high molecular weight. This observation is relevant because it is evident that a substantial fraction of the molecules in a DNA sample can be in the non-Gaussian range (that is, the size, r , is smaller than the molecular weight would indicate and the assumption of Gaussian statistics men-

tioned is compromised). The continuity in this non-Gaussian region of the same hydrodynamic relations observed in the Gaussian range provides the justification for applying the hydrodynamic relations used in the next section to DNA solutions.

Intrinsic viscosity

The viscosity of dilute DNA solutions exceeds that of any other widely studied natural or synthetic substance, and is great enough to be readily measured at the dilutions required for the determination of the intrinsic viscosity. The marked gradient dependence of the relative viscosity, however, prevented for some time the evaluation of the intrinsic viscosity at zero gradient: it is only the zero gradient value that can be used for quantitative interpretations in terms of molecular configuration and size. In 1952 Pouyet obtained a value at essentially zero gradient by use of a Couette viscometer. This value was about 50 in units of 100 cc/g and was more than twice that previously found in the conventional gradient ranges of capillary viscometers. Further developments with the Couette viscometer both in Strasbourg and by Conway and Butler ('54) have shown that various samples of DNA have intrinsic viscosities in the range of 40 to 60.

In our own laboratory, capillary viscometers have been constructed in which 2-m-long capillaries of 1 mm diameter wound as flat helices provide measurements in the range of 30–150 sec⁻¹ mean gradients. These data can then be empirically extrapolated to zero gradient. The value of the intrinsic viscosity so obtained is reproducible to within $\pm 2\%$ and on the basis of limited comparison with Couette viscometers appears to be accurate to within $\pm 5\%$. Values for various carefully prepared DNA from calf thymus fall within the range of 60–72 (Rice and Doty, '57; Simmons, Litt, Zubay, and Doty, unpublished results). If the evidence presented later concerning the near proportionality between intrinsic viscosity and molecular weight is employed here, it is seen that this range could be accounted for by the small variations in mean molecu-

lar weight among the samples. Accepting 66 as the most representative value of the intrinsic viscosity for calf thymus DNA, we examine next the information that can be derived from this.

The quantitative interpretation of intrinsic viscosity is limited to rigid, asymmetric particles that can be treated as ellipsoids of revolution and to randomly coiled polymer chains in which the immobilization of solvent within the polymer coil accounts for the intrinsic viscosity.

Treating DNA as a rod-like particle, Simha's theory leads to an axial ratio of 470 if 0.55 is accepted as the partial specific volume. If 25 Å is taken as the value of minor axis of an ellipsoid equivalent to the Watson-Crick configuration, it is seen that the axial ratio is indicative of an average particle length of 11,700 Å. Flow birefringence measurements interpreted in terms of the same model also lead to calculated lengths of this magnitude. If the Watson-Crick model is used for the mass-to-length ratio, a molecular weight of about 2.3 million can be calculated from this length. However, all these conclusions, despite their self-consistency, must be rejected in view of the angular intensity distribution of scattered light. The latter is quite incompatible with the rod-shaped scattering unit by a very wide margin and no consideration of polydispersity can affect this conclusion.

To interpret the intrinsic viscosity of DNA in terms of a randomly coiled polymer, one has to choose from several theories that, fortunately, lead to fairly similar conclusions. The Flory-Fox relation appears to be the most appropriate one since as it has received the widest testing, has been adapted to include the effects of polydispersity, and has been found to apply equally well to cellulose derivatives that in their low degree of coiling approach the high extension found in DNA (see table 1). The Flory-Fox relation for the intrinsic viscosity, $[\eta]$, is

$$[\eta] = \frac{\Phi}{g \Phi} \frac{r_z^3}{M_w} \quad \Phi = 2.2 \cdot 10^{21} \quad (2)$$

The factor q_{Φ} is equal to unity when the molecular weight of the solute is uniform: its value increases as the molecular weight distribution broadens. Its value for typical distributions can be computed (Hunt *et al.*, '56). For the distribution assumed in the interpretation of light scattering ($M_n:M_w:M_z::1:2:3$) it has the value 1.95. Consequently, if the previously mentioned light-scattering values for r_z (7200 Å) and M_w (8,000,000) are inserted, the predicted intrinsic viscosity is 53—20% less than the observed value. This agreement is however within the range of the uncertainties specified for the experimentally determined quantities. We may consequently conclude that the size and shape of DNA determined by light scattering accounts for the observed viscosity, provided that the assumed distribution of molecular weight approximates the actual distribution.

Sedimentation rate

The sedimenting boundary of a DNA solution is self-sharpening and can therefore be observed in ordinary analytical ultracentrifugation with Schlieren optics down to concentrations of about 0.025 g/dl. These concentrations are too high to ensure accurate extrapolation to the value of the sedimentation rate at infinite dilution, s° . The introduction of ultracentrifuge cells 30 mm thick instead of 12 mm permitted concentrations as low as 0.01 g/dl to be determined. When this possibility was exploited, values of $s_{20,w}^\circ$ were found in the range of 20–23 Svedbergs (Simmons, '55; Peacocke and Schachman, '54; Kawade and Watanabe, '56). Some boundary spreading could be detected at very low concentrations but no estimate of polydispersity could be made from it.

The interpretation of this value is dependent on and limited by the same two hydrodynamic models mentioned in the preceding paragraph. The rigid, ellipsoid-of-revolution model leads to a minor axis dimension of 25–27 Å for the range of 20–23 Svedbergs. If this ellipsoidal model is converted to a cylinder of the same length and volume, the diameter of the

latter would be smaller than the minor axis by a factor of $(2/3)^{0.5}$; that is, the cylinder diameter in this case would be 20.5–22 Å. Although this model is unacceptable for the interpretation of intrinsic viscosity caused by its incompatibility with light scattering, it may be valid for the interpretation of sedimentation rate because the latter is very sensitive to cross-sectional dimensions whereas the intrinsic viscosity is dependent on the axial ratio into which the maximum and minimum dimensions both enter. This point is untested however and the excellent agreement that these dimensions afford with the Watson-Crick model must therefore be viewed with reservations.

The use of the alternative model, the solvent-immobilizing coil, permits the calculation of the sedimentation constant from the light scattering molecular weight and size when the distribution previously discussed is assumed. The relation is

$$s^{\circ} = \frac{q_P M_w}{P \eta_0} \frac{(1 - v\rho)}{N r_z} \quad \text{where } P = 5.1 \quad (3)$$

and substitution into it using 0.55 for the partial specific volume, v , and 1.15 for q_P leads to a sedimentation constant of 18.2 Svedbergs. The difference between this value and that found for the thymus DNA samples on which the light scattering measurements were made (22.5 Svedbergs) is within the limit of uncertainty placed on the values for light scattering. For example, a molecular weight of 9 million and a r_z of 6500 Å would produce complete agreement. The conclusion therefore stands: within experimental uncertainties and making the assumption concerning the molecular weight distribution, it is found that the molecular weight, shape, and size found by light scattering permits the prediction of the observed intrinsic viscosity and sedimentation constant using Flory's relations.

A development in sedimentation that promises to add a new dimension to the characterization of DNA is the bringing into practical operation of an ultraviolet optical system in the Spinco Model E ultracentrifuge permitting the observation of

the sedimenting boundary in DNA solutions at concentrations as low as 0.001 g/dl. This tenfold gain in dilution at which observations can be made makes possible the determination of the distribution of sedimentation constants. This in itself offers a much more discriminating characterization of DNA than the mean sedimentation constant that has been discussed and, in addition, the possibility is presented of determining the molecular weight distribution itself.

For this last step, the relation between sedimentation constant and molecular weight is required (on which some information is given in a later section) and the assumption that the molecules are homologous must be employed, and if possible tested. If, for example, the average size (r) of each DNA molecule did not bear a constant relation to its molecular weight (that is, if the molecules were not homologous) the DNA sample would exhibit a polydispersity with respect to molecular weight and also to configuration. As a result, the molecular weight distribution itself could not be derived from the sedimentation constant distribution.

Professor Schachman, who has pioneered in the development of this method, will present some of his results during the discussion. The other results that are available are due to Shooter and Butler ('56). One of the samples they examined was from our laboratory (SB-11) and had properties similar to the average values used in this paper. It was found that 60% of this sample was contained within the range of 20–30 Svedbergs and that 20% fell below 20 and the other 20% above 30; the mean sedimentation constant was 24. Preliminary considerations indicate that this distribution is not far from that assumed in the foregoing calculations if the assumption of homologous molecules is made. Much careful work will be required before a definitive statement on molecular weight distribution can be made.

Molecular weight derived from intrinsic viscosity and sedimentation constant. By eliminating r_z between equations (2) and (3), we obtain a relation expressing the weight average

molecular weight in terms of the intrinsic viscosity and sedimentation constant.

$$M_w = \left\{ \frac{s^0 [\eta]^{1/3} \eta_0 N}{\Phi^{1/3} q_P \cdot (1 - v\rho)} \right\}^{3/2} \quad (4)$$

This relation has found considerable support when tested in polymeric systems and it has the virtue of not being very sensitive to molecular weight distribution. Nevertheless its application here as in equations (2) and (3) cannot be viewed as a rigorous procedure because DNA is so much more highly extended than the highly coiled type of polymer molecule for which these relations were derived. Rather, it must rest on the observation that these equations have been found to hold for cellulose derivatives that are nearly as extended as DNA (table 1).

Substitution of previously mentioned values for thymus DNA into this relation leads to a value of 12,400,000 for M_w . This value is 50% greater than the light scattering result. The ratio of polydispersity factors ($q_P/q_\Phi^{1/3}$) remains nearly constant (0.90 ± 0.01) over the neighboring range of polydispersity ($M_w/M_n = 1.5$ to 5) and consequently cannot explain any of the discrepancy. It is apparent therefore that these two procedures for deriving the weight average molecular weight are in qualitative but not quantitative agreement. The difference seems to reside either in the inapplicability of equation (4) in a strict sense or in the assumption concerning the molecular weight distribution used in interpreting the light scattering results. It is therefore appropriate that we examine briefly the role of polydispersity in the interpretation of light scattering from DNA.

Polydispersity effects in light scattering from DNA solutions. An analysis of this problem by Benoit, Holtzer, and Doty ('54) has shown that the customary interpretation of the slope and intercept of the reciprocal scattering envelope to give M_w and r_z is valid only if the scattering is observed at

sufficiently low angles, the required angular range decreasing with increasing molecular size. This limitation, however, does not apply if the molecular weight distribution approximates the one in which $M_z:M_w:M_n::3:2:1$. By assuming this distribution in DNA, we avoid consideration of the angular range restriction. But with the evidence in the previous section of a discrepancy between viscosity-sedimentation molecular weight and the light-scattering one, the possible errors that the assumption of a particular distribution may have introduced into the light scattering results must be examined.

The conclusion that can be reached from the investigation of this general problem is that when the lowest scattering angle is 30° the largest radius of gyration (weight average) that can be determined without risk of introducing any error from polydispersity effects is about 600 Å (if the effective wave length of light in solution is about 3200 Å as in the present investigations). Even for this molecular size, only the measurements at angles below 50° could be certain to be free from polydispersity effects. It becomes clear that, with the much larger size of DNA molecules, measurements would have to be made in the angular range of $6\text{--}10^\circ$ to eliminate the possible complications caused by polydispersity. Since there is no likelihood of reaching this range because of practical difficulties, the effects of polydispersity on the present data, which are limited to angles of 30° and above, must be evaluated. This can be done only by selecting hypothetical molecular weight distributions.

Two molecular weight distributions were chosen for this evaluation: one broader ($M_z:M_w:M_n::5:3:1$) and one much narrower ($M_z:M_w:M_n::6:5:4$) than the one previously assumed. The results show that if the true weight average molecular weight were indeed 12.4 million as the viscosity-sedimentation calculation indicated, then the same light scattering data previously employed would have yielded a value of 10.7 million for the apparent molecular weight in the broad distribution and a value of 19.1 million in the narrow distribution.

It is apparent that broadening the distribution brings about a shift of the apparent molecular weight toward that obtained under the previously assumed distribution. A narrowing of the distribution widens the discrepancy. Consequently, the conclusion is reached that a considerably broader distribution than the broad one (5:3:1) investigated here would be required for compatibility with the value of 12.4 million for M_w and the light scattering molecular weight of 8 million.

There is, however, strong evidence against the existence of a molecular weight distribution broad enough to meet this requirement, that is, a distribution for which the ratio M_w/M_n would have to exceed 5. This comes from an experiment designed to see if mild acidic denaturation of DNA was accompanied by any change in molecular weight (Reichmann *et al.*, '54). In this experiment, light-scattering measurements were made on DNA (1) under the usual conditions at neutral pH, (2) at pH 2.6, and (3) after reneutralization. Results of (1) and (3) were essentially identical, showing that the molecular weight had not changed during the exposure to acid. The measurements at pH 2.6, however, showed that the DNA molecules had undergone very considerable contraction with the radius of gyration falling to 880 Å but that the light scattering molecular weight had remained constant. Had the distribution of molecular weight been of the very broad type required ($M_w/M_n > 5$), the light scattering molecular weight under acid conditions should have been nearly 50% lower than that under neutral conditions. Since it was the same, within experimental error, any distribution greatly different from that first assumed (3:2:1) is eliminated.

The result of these rather tedious considerations is support for the first interpretation given of the light scattering data and the nature of the molecular weight distribution that was assumed (3:2:1). On the other hand, the higher estimates of the weight average molecular weight determined from viscosity and sedimentation appears to be in error by as much as 30% (high) and the reason for this apparently resides in a

difference between the hydrodynamic model and DNA itself, either in polydispersity of configuration or in the extreme openness of the DNA structure, which prevents the immobilized solvent from having the spherical symmetry required.

All these considerations represent at best tangential approaches to the fundamental problem of molecular weight distribution. Until sedimentation studies with ultraviolet optics or some other procedure provides more information on this distribution, we are forced to summarize the situation tentatively in this way. The foregoing evidence indicates that the distribution of molecular weights in DNA from calf thymus is such that the ratios of the averages, M_z/M_w and M_w/M_n , lie within about 10–20% of the values of 1.5 and 2 that characterize the assumed distribution. Consequently the light scattering molecular weights may deviate from the true weight average values by this same amount.

DEOXYRIBONUCLEOPROTEIN

In cell nuclei, DNA occurs in close association with basic proteins of quite low molecular weight. This combination, the nucleoprotein, has not been widely studied by physical methods because attempts to bring it into solution in discrete particles have not been successful. Instead gel-like aggregates having very high viscosity result. Salt added to the aqueous suspension causes precipitation in the vicinity of 0.15 *M* NaCl and at higher concentrations dissolution occurs again but the complex is now dissociated into DNA and protein.

Within the last few months the nucleoprotein from calf thymus has been isolated in solution in completely dispersed form (Doty and Zubay, '56) and since this may provide the basis of an examination of the nucleoprotein in a comparable way to that in which DNA has been studied, a brief description may be of interest.

By avoiding initial extractions of protein material in 0.15 *M* NaCl and indeed by keeping the ionic strength at 0.0007 *M* throughout the isolation, we found a means of obtaining a

true solution of what appeared to be nucleoprotein particles. A preliminary examination of this material shows its molecular weight by light scattering to be 19 ± 5 million. It is composed of nearly equal weights of protein and nucleic acid and, upon being passed through the normal preparative procedure for DNA, yields samples having the same characteristics as those described in earlier sections. It has somewhat smaller dimensions than DNA: r_z has a value of 4200 Å. The intrinsic viscosity is 35 and the sedimentation constant is 50 Svedbergs.

These observations indicate that the nucleoprotein is composed of single DNA molecules and an equivalent weight of histone and that its radius of gyration is only a little more than half that of the DNA. Thus the histone appears to cause a somewhat tighter coiling of the DNA. The sedimentation constant is indicative of a larger cross-sectional diameter. The use of equation (4) to compute M_{sc} leads to values twice that of light scattering in comparison with the 50% higher value produced for DNA itself. The larger difference here may actually find its origin in the effect that a minor amount of aggregated material might have on the sedimentation constant.

The nucleohistone prepared in this manner can be obtained in high yields from nuclear fragments and it appears therefore that the material on which these preliminary studies have been made may be relatively undamaged building blocks of the chromosomes.

THE DENATURATION OF DNA

The hydrogen bonds connecting the two polynucleotide strands in DNA can be broken by extremes of pH, heat, and the addition of strong agents for breaking hydrogen bonds. Since the hydrogen bonds are arranged periodically, it is expected that they will melt out in a cooperative manner; that is, a large number will break in one step and this will have a high energy of activation and will occur within a narrow temperature range. This is generally found to be the case and

as a consequence the term denaturation appears justified. Limitations of space require that the documentation of this behavior be very brief.

If the pH is lowered gradually by dialysis in the presence of $0.2\ M$ NaCl, the intrinsic viscosity remains at its normal high value until about $pH\ 3.2$. Between $pH\ 3.2$ and 2.6 it undergoes a drastic fall, as shown in figure 3. As mentioned earlier, no

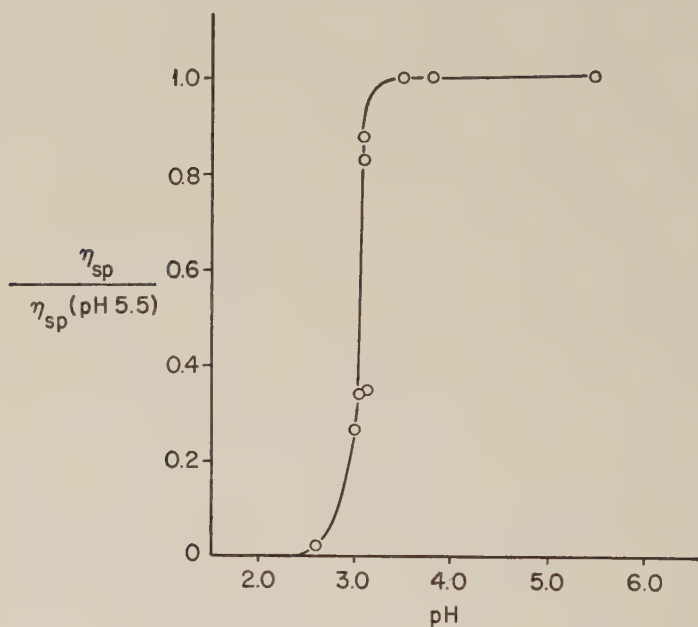


Fig. 3 The change in the specific viscosity of DNA in $0.2\ M$ NaCl (0.01%) upon lowering the pH . (C. A. Thomas, Thesis, Harvard University, 1955.)

change in molecular weight appears to occur if the exposure to $pH\ 2.6$ is brief. Upon prompt reneutralization, the molecular configuration returns to the original one. The interpretation of this is the following: as the proton concentration is gradually increased, the point is reached where the acidic groups participating in the hydrogen bonds open up and accept the protons in a cooperative manner. In the pH range indicated, however, not all the groups are titrated, and as a

consequence some hydrogen bonds remain intact. Except for these occasional regions of intact hydrogen bonds the polynucleotide chains are free to take up a randomly coiled and flexible configuration, with the result that there is a very great over-all contraction of the DNA molecule and a consequent

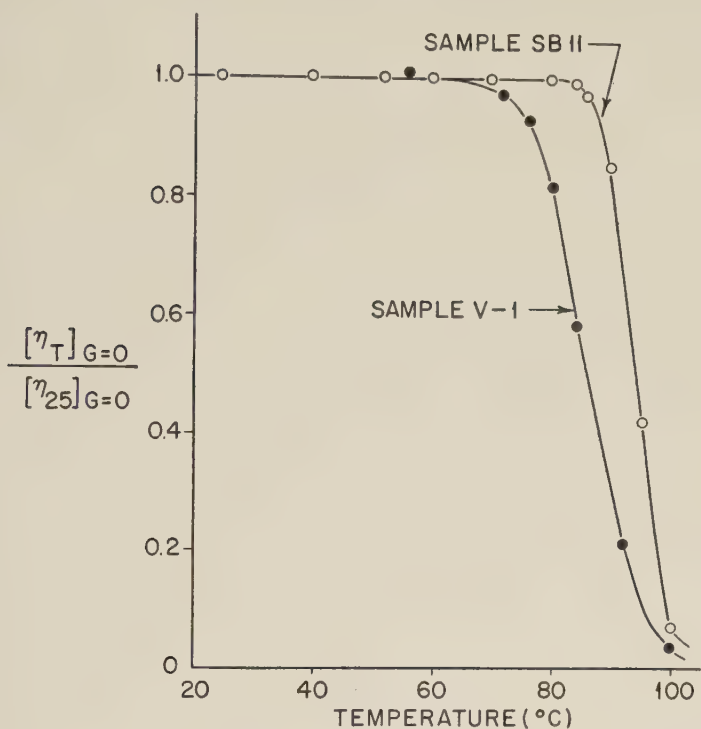


Fig. 4 The intrinsic viscosity of DNA as a function of the temperature to which the DNA solution had been heated for 1 hour. The solvent is 0.015 *M* sodium citrate and 0.15 *M* sodium chloride. (From Rice and Doty, '57.)

drop in viscosity. The unbroken hydrogen bonds maintain the two polynucleotide chains in register; upon reneutralization the hydrogen bonds can then re-form between the identical base pairs that existed in the original state. Prolonged exposure at pH 2.6 results in the removal of purine groups and it appears likely that this is the cause of the loss in reversibility (Thomas and Doty, '56). In alkaline pH this same set of observations

can be repeated. The role played by pH 2.6 on the acid side is taken by pH 11.8 on the alkaline side (P. Ehrlich and Doty, unpublished results).

The thermally induced breakdown of the hydrogen bonding is the direct analogue to the melting out of a crystallite and this can be observed simply on heating a DNA solution. The viscosity drops sharply in a narrow temperature range whose location depends on the ionic strength and the character of the sample. This is shown in figure 4, where the intrinsic viscosity measured at room temperature after a 1-hour exposure to the temperature indicated on the abscissa is plotted relative to its room temperature value before heating; that is, 66. No change is observed until about $70^{\circ}C$. for one sample and $86^{\circ}C$. for the other. The viscosity falls considerably at temperatures above these threshold values and by 100° has reached about a 20-fold lowering. The temperature at which the viscosity has fallen in half is 86° and $94^{\circ}C$. for these two samples: this may be conveniently referred to as the denaturation temperature. Current work indicates that the denaturation temperature under standardized conditions plays the role of a melting point in organic chemistry: that is, the higher it is, the purer the substance. In DNA, the lowering below the maximum value has been shown by Zamenhof to arise from a variety of mis-treatments of the DNA sample, such as limited enzymic attack or previous thermal treatment. The sample with the higher denaturation temperature in figure 4 is representative of the best material so far examined.

Light-scattering studies again show no change in molecular weight until temperatures of nearly $100^{\circ}C$. are employed (Rice and Doty, '57). The thermally induced denaturation is very similar to that brought about by acid except that only the irreversible part has been observed in these studies since observations were made after cooling rather than at the temperature of exposure.

The effect of adding denaturing agents to the solution can be seen by repeating the viscosity-temperature experiment in

their presence. Figure 5 shows measurements of this kind in the presence of urea and other agents. The denaturation temperature is lowered by as much as 17°C . with $8\text{ }M$ urea present. All these experiments have been carried out in the presence of $0.15\text{ }M$ NaCl and $0.015\text{ }M$ sodium citrate. Lowering the ionic

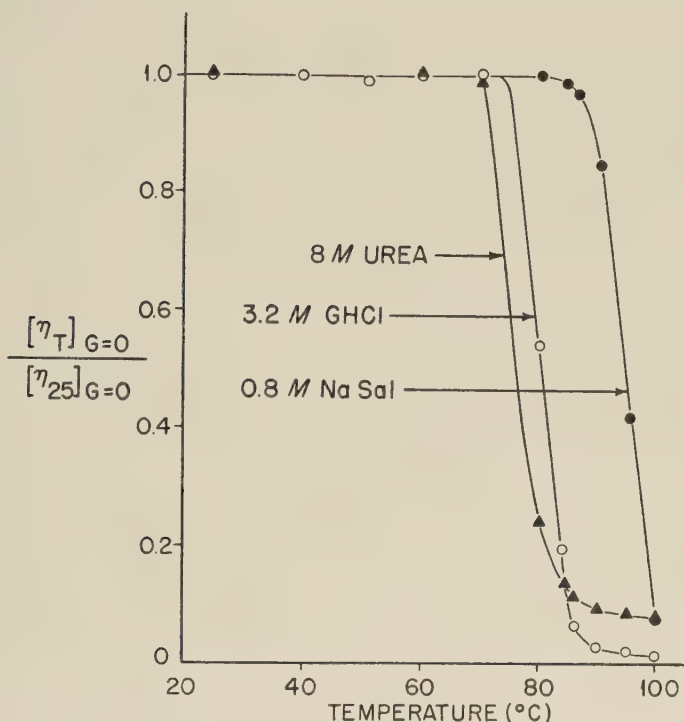


Fig. 5 The effect of denaturing agents on the thermal denaturation of DNA as observed in terms of the intrinsic viscosity after heating for 1 hour at various temperatures. Sample SB11: $0.15\text{ }M$ NaCl and $0.015\text{ }M$ sodium citrate was present in all cases. (From Rice and Doty, '57.)

strength brings about a lowering of the denaturation temperature. This is undoubtedly caused by the weakening of the helical structure as a result of unshielding the negatively charged phosphate groups with the consequent increase in the electrostatic repulsion between them.

Thus far the study of thermal denaturation has opened up at least as many questions as it has answered. For example, there are indications that the melting out of the hydrogen bonds occurs in regions only 20–100 nucleotides in length. What causes the process to stop when this size gap is produced is not known. If all the hydrogen bonds could be broken, the two chains should come apart in the form of simple, randomly coiled, polynucleotide chains. Despite some claims that this has been accomplished, it is our belief that it has not yet been achieved. Why it has not been achieved remains a question. Perhaps disorganized hydrogen bonds re-form in part after the regular structure is melted out and it is these that keep the polynucleotide strands paired.

DEGRADATION OF UNDENATURED DNA

Although the over-all problem of the degradation of DNA with its attendant chemical problems is a vast subject indeed, it appears that the degradation that can be brought about while keeping the hydrogen-bonded structure intact is at present quite limited. There are two ways of bringing this about: limited enzymic attack and sonic treatment. Thomas ('56) following early leads obtained by Reichmann ('56), and Schumaker and associates ('56), following earlier leads indicated in the work of Dekker and Schachman ('54), have shown independently that deoxyribonuclease makes only single chain scissions in the double-stranded structure. Within a rather large uncertainty these appear to occur at random. As a consequence, the molecular weight does not fall until two cuts occur either opposite each other or within about two nucleotides of such a point. Indeed the viscosity does not show a significant decrease until about 100 phosphate-ester bonds have been cleaved. Thus enzymic damage can remain hidden until more specific tests such as the determination of the denaturation temperature reveal the hidden defects.

Physical-chemical studies of DNA have been handicapped by not having homologous samples of substantially different

molecular weight. Ordinarily such samples might be expected from fractionation but the fractionation of DNA with respect to molecular weight has not yet proved successful in this regard. As a result of this state of affairs, the sonic degradation of DNA was undertaken (B. H. Bunce, P. Doty, and S. A. Rice, unpublished results). Exposure of very dilute, oxygen-free solutions in a 9-kilocycle generator for periods of 1 minute to 1 hour produced material over the molecular weight range of 3,000,000 to 300,000. The remarkable feature of these products was that the denaturation temperature was unchanged. This was interpreted as a strong indication that the hydrogen bonding has remained unaffected and that the sonic vibrations have merely introduced double chain scissions each one of which leads to a fall in molecular weight.

These fractions provide the basis of a number of studies of interest. Already it has been shown that the intrinsic viscosity of these samples is proportional to the 1.1 power of the molecular weight and the sedimentation constant is proportional to the 0.36 power of the molecular weight. Assuming that the DNA molecules are homologous, these results offer a means of interpreting the sedimentation distribution obtained from ultracentrifugal observations in which ultraviolet optics were used as previously indicated. The production of these fragments of DNA also opens up the possibility of investigating their role in bacterial transformation: this point is now under study.

Despite the length of this review it would be improper to imply that it has been comprehensive or well balanced: indeed it obviously reflects a nearly total preoccupation with the studies of our own laboratory. But it is, I think, representative of the present state of the elucidation of the detailed structure and properties of DNA as revealed by physical-chemical methods. Finally, I must express the appreciation of my co-workers and myself for the collaboration we have enjoyed with Dr. Norman S. Simmons of the University of California at Los Angeles and the support that has been provided by the

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research grant (C-2170) for the National Cancer Institute of the National Institutes of Health, Public Health Service, and other grants from the Institutional Grant to Harvard University from the American Cancer Society.

GENERAL DISCUSSION

SINGER¹: In a very low solute concentration, one may encounter a convection effect that would be very hard to pick up by conventional techniques and that would have the effect of spreading the boundary. With bacteriophage at very low concentrations, in the ultracentrifuge, we followed the shape of the boundary by infectivity measurements with the bacteriophage and found, at concentrations too low to be measured optically, a spreading presumably caused by the instability of the boundary. Under these circumstances, might this be an effect?

SCHACHMAN²: It certainly is an effect. I think the major difference is that when you run phage in those experiments you run at low speed and because it is so big the stabilizing effect of the centrifuge field is, indeed, very small. Convection is very hard to get at high speed. But if we make a boundary of DNA in centrifuge we will lose the boundary fields at 1000 or 2000%. If we run at the high speed we will not lose the boundary. One can run materials at high speeds that one cannot run at low speeds.

SINGER: That might depend on the particular material and a lot of individual considerations. It is just something to worry about a little.

RANDALL³: I wish to review our knowledge of the structure of DNA, with particular reference to the contributions made by my colleague, M. H. F. Wilkins and his associates, and to refer to the implications of this structural investigation in various directions.

¹ S. J. Singer, Yale University.

² Howard K. Schachman, University of California.

³ J. T. Randall, University of London King's College.

Background knowledge

1. Chemical study has shown that the nucleosides are linked by 3',5'-phosphodiester groupings in both DNA and RNA.

2. Titration experiments have indicated a large amount of intramolecular H bonding between bases in DNA.

3. Analysis of DNA has shown that, although the ratio of adenine to guanine could vary considerably with the species of origin, there were always equal proportions of (adenine + thymine) and (guanine + cytosine).

4. Physical studies demonstrated that DNA molecules were long, threadlike molecules with the planes of the bases tending to lie perpendicular to the length of the molecule.

5. Fibers of DNA gave excellent X-ray diffraction photographs and these, together with chemical data, have enabled the structure of DNA, i.e., its three-dimensional configuration, to be established with reasonable certainty.

6. RNA gives inferior X-ray data and its structure has not yet been elucidated to anything like the same extent.

X-ray studies. Early DNA experiments by Wilkins and his colleagues suggested a helical structure and provided the main dimensions of the helix. About this time important advances were made by Watson and Crick, who built a molecular model of DNA. Factors that guided the building of the model were: (1) the X-ray data from our laboratory provided the dimensions of the helix and showed that the molecule consisted of two or more polynucleotide chains; (2) the base analysis data suggested that the bases occurred in pairs; (3) titration studies suggested that the polynucleotide chains were joined by H bonds between bases.

Watson and Crick made the important assumption that the number of chains (two) and the ingenious manner of their base pairing resulted in both pairs being equivalent and symmetrical with regard to the joining of the two phosphoester chains. The scheme was attractive because it explained how

DNA, consisting of four different bases, could exist as a molecule of great regularity that crystallized with considerable perfection irrespective of the ratio adenine/guanine. The structure was somewhat hypothetical, and was not derived entirely from experiment. It was found in fact that the model was not in adequate agreement with the detailed data, and it remained to be shown that a model of this kind could be built that would agree well with the data.

In 1955 my colleagues described a structure that was in satisfactory agreement. Since then, further work has shown that the correctness of the structure has been established with reasonable certainty. Lithium, potassium, and rubidium salts prepared by L. D. Hamilton and others of the Sloan-Kettering Institute have been especially useful.

With the lithium salt, which contains fewer electrons than sodium, the contribution of the lithium ion can be ignored.

The lithium salt crystallizes with the DNA molecule in the normal, somewhat extended, state. Sodium, potassium, and rubidium salts crystallize in a compressed configuration.

Evidence for two polynucleotide chains and possibility of molecules in other forms. The strongest evidence for two chains is that the X-ray data agree well with a two-chain model; but the result is not without ambiguity. Density and water content of DNA fibers support the two-chain model, but results are rather inconsistent. DNA fibers contain $\sim 50\%$ of amorphous material that sometimes has a higher density and a lower water content than the crystalline regions studied by X-ray diffraction.

No completely convincing evidence is yet available. The possibility remains that an appreciable fraction of DNA may exist in other forms. Titration and base analysis data strongly suggest that a large part of DNA contains H-bonded base pairs, but it must be stressed that X-ray data refer only to regularly arranged molecules.

Structure of deoxyribonucleoproteins. It is hoped that such a study may throw light on the functional relation of nucleic

acids and proteins. The simplest basic protein to combine with DNA is of the protamine class, and Wilkins and others have shown the mode of combination.

The polypeptide chain in nucleoprotamine is in a fully extended form and winds over the smaller of the two grooves round the DNA molecule. The side chains extend at right angles to the polypeptide chain so that their basic end groups are held electrostatically to the phosphate groups of the DNA. One-third of the residues in protamines are nonbasic and these probably occur at folds in the chain, so that all the basic groups are able to combine with the phosphate groups. A single residue of the chain cannot form a fold, but two can. Sequence analysis shows that the nonbasic residues occur in pairs (Felix). It is not known whether the folds point inward toward the nitrogen bases, or outward.

So far as is known, protamines occur only in certain sperm heads.

In cells that multiply or synthesize protein, DNA appears always to be combined with histone and is somehow in association with nonbasic protein and possibly RNA in chromosomes.

Some nuclei consist largely of nucleohistone, and X-ray diffraction studies give the same results on these whole nuclei as on water-extracted nucleohistone. Characteristic molecular morphogenesis may be preserved intact during extraction. The extracted material may be stretched into fibers in which a fraction of the molecules are parallel to the fiber length. The X-ray diffraction photographs are not so well defined as those from DNA alone.

The wide-angle picture is like a poor B-type picture of DNA showing (1) the helix and (2) that it is not the same as nucleoprotamine since it has no strong first layer line. It is, however, very likely that most DNA phosphate groups are combined with basic groups on histone because sodium and potassium analysis of nucleohistone shows there is only about one metal ion per four phosphates.

We also know that the lysine-rich fraction of histone (Mirsky) can combine *in vitro* with DNA to give a compound that X-ray diffraction shows to be similar to nucleoprotamine. However, since histone, unlike protamine, consists of many fractions of widely differing composition, it may not be a simple matter to find how it combines with DNA. The outer regions of the X-ray diffraction pattern of nucleohistone are very similar to that of DNA alone and this result is compatible with various arrangements of the histone. The histone might be wrapped around both grooves on the DNA molecule with the nonbasic residues pointing inward toward the nitrogen bases. Alternatively, the polypeptide chains might be arranged in some complicated and irregular manner so that the basic side chains reach the phosphate groups. It is not impossible that all the histone may be packed in the deep groove on the DNA molecule. I think, however, that the first suggestion is more likely. So far, there is little sign of an α -helix configuration existing in the histone.

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THE FORCES BETWEEN PROTEIN MOLECULES IN SOLUTION: A SUMMARY¹

JOHN G. KIRKWOOD

*Sterling Chemistry Laboratory, Yale University,
New Haven, Connecticut*

Until there is convincing evidence to the contrary, it is reasonable to accept the hypothesis that the forces between protein molecules are of the same nature as those acting between simple molecules of low molecular weight. At small intermolecular distances, quantum mechanical exchange produces a repulsion that determines the size and shape of the molecules. At larger distances, the London dispersion forces act to produce a general van der Waals attraction. Moreover, since proteins are amphoteric polymers of the highly polar amino acids, many of which possess acidic or basic side chains, the molecules undoubtedly possess characteristic distributions of electric charge that give rise to strong electrostatic interactions. Evidence for the dominant role of electrostatic forces is provided by the sensitivity of the thermodynamic interaction of protein molecules to ionic strength. The reduction in interaction with increasing ionic strength, frequently observed, is produced by the screening action of the statistical space charge of the electrolytic environment. It is easy to demonstrate that such screening could be effective only on that part of the interaction that is electrostatic in origin and not on the high-frequency exchange forces and van der Waals forces.

Although of the same basic origin as those between simple molecules, the forces between protein molecules possess special features arising from their complex structural organ-

¹Summary of lecture given also at a Symposium on "Molecular Structure and Biological Specificity," Washington, D. C., October 1955; sponsored by the American Institute of Biological Sciences and supported by the Office of Naval Research.

ization. These special features relate to the pattern of arrangement of the structural elements responsible for the specificity of interaction and to the mobility of the charges responsible for electrostatic interaction. The special forces arising from the mobility of the charge distributions have received theoretical treatment by Kirkwood and Shumaker ('52). They will be the principal subject of this discussion. Proteins, considered as ampholites, contain a large number of neutral and negatively charged basic groups, for example NH_2 and COO^- , to which protons are bound to a degree determined by the $p\text{H}$. Except in highly acid solutions, the number of basic sites generally exceeds the number of protons bound to the molecule so that there exist many possible configurations of the protons, differing little in free energy, among which fluctuations induced by thermal motion may occur. Fluctuations in the number and configuration of the mobile protons impart to the molecules fluctuating charges and fluctuating electric multipole moments. Let us consider two protein molecules in fixed orientation separated by a distance R . As the result of fluctuations in charge distribution, associated with the Brownian motion of the mobile protons, each molecule produces an alternating electric field at the point of location of its neighbor. These alternating electric fields produce in turn a mutual electrical polarization of the average proton distributions on the two molecules. When averaged over a time long relative to the periods of Brownian motion, this polarization gives rise to the supplementary attractive force between the two molecules with a potential diminishing asymptotically as $1/R^2$. In the presence of an electrolytic environment, the long range of this force is substantially diminished by Debye-Hückel screening. Fluctuations in charge and charge configuration associated with bound ions other than protons also make a contribution to this special type of intermolecular force.

It is not in general possible to distinguish between the fluctuating and static electrical interaction of protein mol-

ecules by thermodynamic measurements. But that part of the fluctuating force arising from total charge fluctuation in a salt-free isoionic solution may be isolated from effects caused by all other intermolecular forces since it gives rise to a term in the excess chemical potential proportional to the square root of the protein concentration. All other intermolecular forces, both van der Waals forces and electrostatic forces associated with permanent and fluctuating multipoles, contribute only terms proportional to the first and higher powers of the concentration. Timasheff *et al.* ('55) measured the excess chemical potential and activity coefficient of isoionic bovine serum albumin by the well-known technique of light scattering. Their results verify the prediction of the theory of Kirkwood and Shumaker that the excess chemical potential should decrease asymptotically with the square root of protein concentration at high dilutions, as a consequence of the long-range interaction produced by fluctuations in total charge. They determine the value of 3.5 protonic units for the root-mean-square charge fluctuation of a molecule of BSA in isoionic solutions. This value is in excellent agreement with value 3.4 calculated from the titration data of Tanford. Similar measurements, to be reported later, were carried out on human serum mercaptalbumin and bovine serum mercaptoalbumin. The results may be quantitatively interpreted by means of the charge fluctuation theory.

Kirkwood ('55) has used the concept of interaction through charge fluctuations to provide an interesting explanation for the participation of the protein moiety of an enzyme molecule in the mechanism of hydrolytic enzyme reactions. If it is supposed that the catalytic site of the enzyme is situated at the center of a constellation of vicinal basic groups, and if there is a substantial increase in dipole moment in the activation of the catalytic site-substrate complex to its transition state, interaction by the fluctuation mechanism of the basic groups and their attached protons with the dipole moment of the activated complex, can substantially diminish the free energy of activation, in a predictable manner dependent on

pH . One of the interesting features of this interaction is that it passes through a maximum at a pH equal to the pK of the conjugate acids of the participating basic groups. A consistent analysis of the pH dependence of the rates of hydrolysis of esters and amides by a group of representative enzymes has been achieved by the theory.

It seems reasonable to predict that the role of the fluctuation force will turn out to be important and, in certain instances, decisive in many other examples of interaction between protein molecules in solution. It is clear that highly specific interactions might well arise from the fluctuation mechanism if the concept of complementary patterns is invoked. In favorable orientations, steric matching of a constellation of basic groups on one molecule with a complementary constellation on the other could well produce a redistribution of protons leading to a strong specific interaction, depending on the local structural details of the complementary constellations. Considerations relating to the specificity of the fluctuation force, as in other types of interaction, must necessarily remain speculative until more detailed knowledge of the fine structure of proteins is available.

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FORMATION AND PATTERNS OF CELLULOSE FIBRILS: A SUMMARY

A. FREY-WYSSLING

*Head of the Laboratory of General Botany,
Swiss Federal Institute of Technology,
Zürich, Switzerland*

FOUR FIGURES

In contrast to the striated protein fibers, the submicroscopic cellulose fibrils in the plant cell walls are smooth. They consist of parallelized chain molecules of cellulose. The symmetry of these macromolecules is governed by a twofold screw axis, which causes stretched chains. This facilitates their crystallization into a chain lattice. The individual chains are tied together by hydrogen bonds that are active in two almost perpendicular directions. In one of these, owing to a smaller distance of the oxygen atoms (2.5 Å), the hydrogen bonds are somewhat stronger than in the other direction (2.8 Å). As a result, the filiform crystallites or micellar strands have a more or less flattened shape; cross sections of 50×60 to 30×100 Å² have been reported. Since the area required by an individual cellulose chain is 33 Å², such an elementary fibril holds about 100 chain molecules. Its crystalline core is coated by less orderly arranged so-called paracrystalline cellulose chains, which cause an aggregation of the elementary fibrils to microfibrils with a diameter of about 250 Å. If these are formed in short-range distances, they have a tendency to fuse together in the plane of the strongest hydrogen bonds, which coincides with the cytoplasmic surface. The defective crystallinity of the microfibrils is caused by the liberation of water molecules during

the crystallization; since this water cannot escape quantitatively, it is partly occluded.

The fused microfibrils compose the microscopic layers of the secondary plant cell wall. These lamellae can be mechanically split into microscopic fibrils that, in the cotton hair, have a diameter of about $0.4\ \mu$. Considering that such hairs are giant cells with macroscopic dimensions, the cotton fiber is, to my knowledge, the only biological object whose fine structure is completely known — from the macroscopic hair with

TABLE 1
Fine structure of a cotton hair

	STRUCTURAL ELEMENT	DIAMETERS	AREA OF CROSS SECTION	NO. OF CHAIN MOLECULES
Macroscopic	Cotton hair	$20\ \mu$	$314\ \mu^2$	750,000,000
Microscopic	Fibril	$0.4\ \mu$	$0.16\ \mu^2$	500,000
Submicroscopic	Microfibril	250 A	$62,500\ \text{A}^2$	2,000
	Elementary fibril	$50 \times 60\ \text{A}$	$3,000\ \text{A}^2$	100
Amicroscopic	Chain molecule	$8.3 \times 4\ \text{A}$	$33\ \text{A}^2$	1

its helically arranged microscopic fibrils through the submicroscopic range with its microfibrils consisting of very fine crystalline strands and down to the amicroscopic macromolecular chains whose chemical structure is entirely known. Table 1 gives a survey of these relations.

The fibrillar patterns of the plant cell walls that we observe in the electron microscope are composed of microfibrils. The plasmalemma of the cytoplasmic surface produces them in a matrix of amorphous pectins (polygalacturonides). In this way a plastic gel reinforced by cellulose fibrils is formed that shows a striking analogy to the connective tissue where collagen fibers are embedded into an amorphous mass of hyalouronides.

The cellulose of the microfibrils polymerizes and crystallizes simultaneously *in situ*. Every cellulose chain consists of several thousands of glucose residues, each of which is 5 Å long, resulting in a chain 1–3 μ long. Since these chains run antiparallel in the lattice, it seems very unlikely that the growth of the crystalline lattice is provided by the apposition of individual chain molecules, because their diffusibility would be almost nil. Apparently, the microfibrils originate at once in their total length; no tip growth has yet been observed. This statement is important because the microfibrils appear to be interwoven. In nature, the woven texture is not so dense as it seems in the electron microscope, because the whole pectic matrix is removed by the preparation process.

In very young meristematic and cambial cells, the textural threads of the so-called primary wall are evenly dispersed. In certain cell types, as a first differentiation, primary pit fields are formed by putting the uniformly woven microfibrils locally apart (parenchyma cells, tracheids). If many such fields are opened a surface growth results (mosaic growth). Other cell types do not show any open fields (laticifers). As a second phase, the cytoplasm secretes a new net of microfibrils onto the existing one. In the pit fields the additional microfibrils are gathered into coarser strands that are arranged around submicroscopic pores. In this way pit membranes with their plasmodesmata are formed. The areas between the pits increase in surface by stretching the original texture, which then is covered by a new membrane (multinet growth).

This multinet growth is especially conspicuous in the so-called tip growth of hairs, pollen tubes, and hyphae.

When the primary wall is full grown, it is consolidated by the apposition of parallelized microfibrils that form the secondary wall. Along the edges of meristematic cells, the secondary wall can appear very early, but it does not prevent a further extension growth because these reinforcements have still a noticeable plasticity.

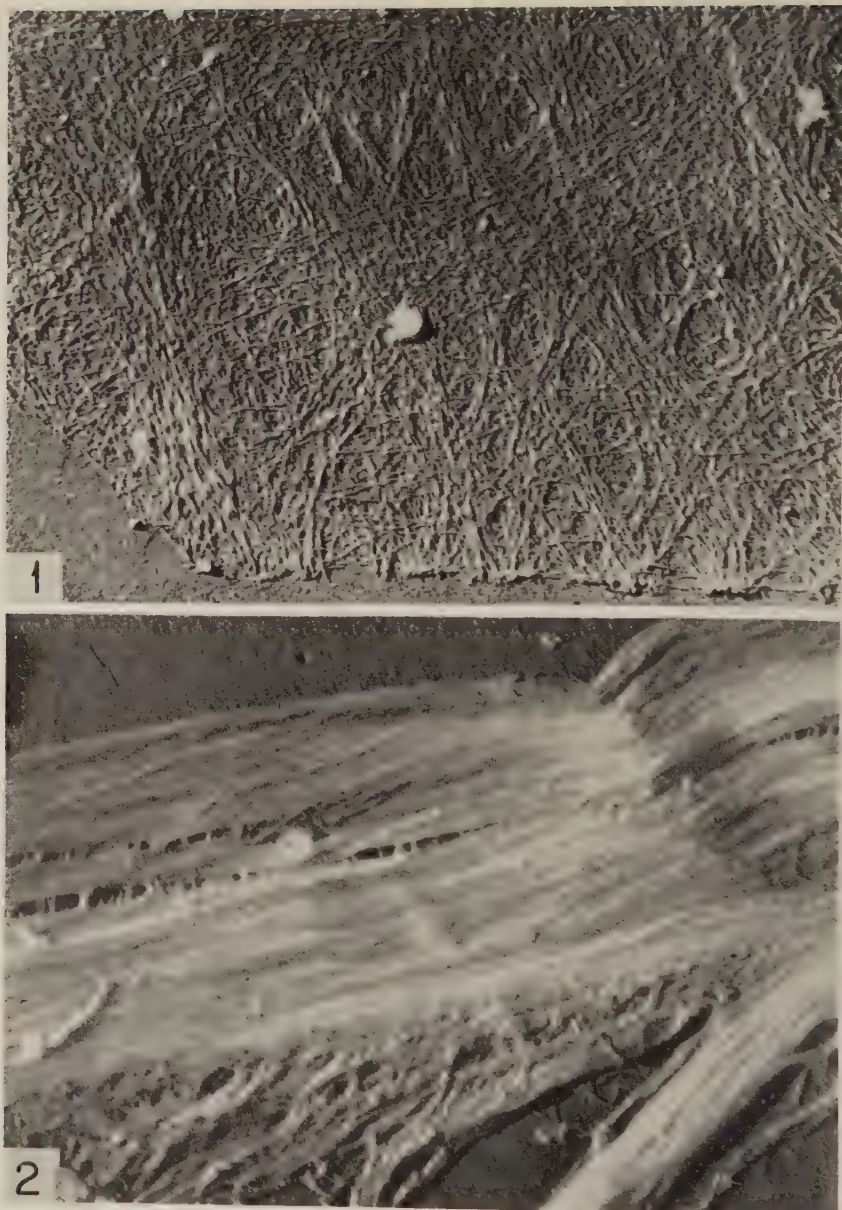


Fig. 1 Primary cell wall of a parenchyma cell in the root cortex of *Zea Mays*. Primary pit fields are visible. (Photo. by H. Stecher: 14,000 \times ; $1\mu = 1.4\text{ cm.}$)

Fig. 2 Secondary cell wall of the cotton hair. The deviation of the parallelized microfibrils is a so-called slip-plane. (Photo. by K. Mühlethaler: 41,000 \times ; $1\mu = 4.1\text{ cm.}$)



Fig. 3 Cutinized membrane of the pollen tube of *Tulipa*. (Photo. by A. Vogel: 32,500 \times ; $1\mu = 3.25\text{ cm.}$)

Fig. 4. Membrane of the pollen tube after removal of the cutin. (Photo. by A. Vogel: 32,500 \times ; $1\mu = 3.25\text{ cm.}$)

As a result of these investigations, it follows that there is no proper intussusception of cellulose fibrils during the surface growth of the plant cell wall. There is, rather, a continuous shedding of its surface lamellae that are stretched by the growth in area and uninterruptedly replaced by new woven nets. On the other hand, the loosening of the texture in the primary pit fields and the gathering of individual microfibrils into coarser strands during the differentiation of the pit membrane of simple or bordered pits, show that some kind of intussusception must occur by the growth of either the cytoplasm or the pectic matrix between the cellulosic microfibrils. These differentiations in confined areas prove that there is no all-around growth in the plant cell, but that the cytoplasm is capable of inducing local growth phenomena that cannot be explained as a passive stretch by the hydrostatic turgor pressure. Therefore, the growth of cell walls must be considered as the result of localized dynamic activities of the cytoplasm.

This paper will be published in extenso with pictures and bibliography as one of the Prather Lectures 1955-56 by the Harvard University Press.

GENERAL DISCUSSION

JEHLE¹: You have shown some pictures of the orientation of cellulose macromolecules. Could you tell us some more details about these orientations in all three directions? How are molecules orientated with respect to each other in the crystal lattice expressing orientations by means of Cartesian X, Y, Z axes?

FREY-WYSSLING (explaining at the blackboard): This is the unit cell and you find that the rings of the glucose residues of the cellulose chains are arranged in parallel planes. The unit cell has four glucose residues belonging to two different chain molecules; one of them is orientated in this direction and the other points in the opposite direction (antiparallel arrangement).

¹ Herbert Jehle, University of Nebraska.

JEHLE: What about two other directions on the cross section?

FREY-WYSSLING: If the microfibrils are laid down, the surface of their ribbon-like shape coincides with the surface of the cytoplasm. But the surface of the glucose rings takes a diagonal position under about 45° to the surface of the cell wall.

JEHLE: One of the pictures showed an orientation like this one (pointing his two forefingers parallel in opposite directions). Is that correct?

FREY-WYSSLING: Yes; it is the result of the antiparallel orientation of the cellulose chains. One chain goes up in this way and comes in the primary alcohol near the top of the glucose rings, while the next runs down showing its primary alcohol near the bottom of the ring.

JEHLE: That is very interesting because that is exactly the most advantageous London-van der Waals orientation.

PHYSICAL-CHEMICAL STUDIES ON DEOXYRIBONUCLEIC ACID ¹

A DISCUSSION

H. K. SCHACHMAN

*Biochemistry and Virus Laboratory, University
of California, Berkeley*

THREE FIGURES

It is rather difficult for me to have anticipated everything that Doctor Doty was going to talk about; and, not having had the pleasure of attending any of the Oak Ridge Symposia, I have no precedence on which to base my activities in the role of a discussant. I will try to arrange my remarks in an order corresponding to Doty's talk.

When J. D. Watson and F. H. C. Crick first proposed their exciting structural model for deoxyribonucleic acid (DNA), we thought it should be possible to obtain information to test this model from a study of the kinetics of the enzymic degradation of DNA. Since the enzymologists have not yet found much specificity for deoxyribonuclease (DNase), we hope that the enzyme is behaving the way *we* want it to behave — namely, that it is breaking only phosphodiester bonds, one at a time and also indiscriminately. There is cause for concern here because only about one in every five bonds is broken after complete digestion by the enzyme, and yet we are assuming that the enzyme breaks bonds at random.

We would like to examine the decrease in molecular weight as a function of time of enzyme treatment and to determine when and how the molecule begins to fall apart. Our data

¹The work reported here has been supported by a grant from the National Science Foundation and has been performed largely with V. N. Schumaker and E. Glen Richards.

of a year ago show that the decrease in viscosity is very rapid, the decrease in sedimentation coefficient measured at two different concentrations is much less rapid, and for a long period of time there is no change at all in the optical density of the solution at 260 m μ .

As Doty has indicated, many workers (particularly R. Thomas and L. F. Cavalieri) have shown that the ultraviolet absorption of DNA is less than that calculated from the sum of the absorptions of the constituent nucleotides. It is also well known that the optical density increases to the calculated amount whenever DNA is subjected to treatments that break down its organized structure. Alkali, acid, heat, or enzymic digestion will cause this increase in absorption, known as the "hyperchromic effect." Of interest to us is the observation in our kinetic studies that the optical density remains constant even though the molecular weight decreases from about 6×10^6 to 10^5 . Different explanations have been proposed to account for the "hypochromicity" of DNA and we need not go into this matter here, except to say that the capacity of DNA or partially degraded DNA to exhibit its hyperchromic effect is a measure of intact molecular organization involving the absorbing purine and pyrimidine bases. Thus we can imagine the structure as a pair of railroad tracks held together by railroad ties, which are the hydrogen bonds. The enzyme is acting like a pair of scissors cutting the individual tracks, occasionally with breaks exactly opposite each other or only slightly staggered. If the attacks in the two chains are only slightly staggered, the molecule is split into two parts. When the molecular weight is about 100,000, there are already breaks in each of the tracks. The chains are becoming short enough that the hydrogen bonds holding them together can no longer compete with thermal energy, and the two chains begin to separate, breaking down the molecular organization and leading to an increase in optical density. By the time the molecular weight had reached about 30,000, the optical density attained its final value, indicating that the strands had become completely separated.

Since these early studies, we have gone back to the first stages of the reaction and stretched out the curve by working at lower enzyme concentrations. We did this to find out whether the enzyme must break two bonds almost opposite each other before there is a scission of the macromolecule, as revealed by a decrease in the molecular weight. The necessary theory was derived and the experiments performed with V. N. Schumaker and E. G. Richards at Berkeley. The degradation was followed by viscosity measurements in conjunction with ultracentrifugation, rather than by light scattering, since we have frequently observed aggregates in the ultracentrifuge. These aggregated particles are likely to cause more difficulty with the interpretation of light-scattering measurements. Viscosity measurements were made at three different shear gradients and the data extrapolated to zero shear gradient. We have used a linear extrapolation, which probably leads to values that are too high early in the study, and some doubt may be raised at this point. In any case, we now have the viscosity as a function of time of enzyme treatment. If we now plot $\log (1-R)$ versus $\log t$, where R is the ratio of the viscosity at time, t , relative to the initial viscosity, we get a straight line whose slope gives an indication of the number of properly arranged attacks necessary to cause a scission in the macromolecule. In other words, the slope of this curve should give the number of strands. When we make this plot, and I might say that we can use time in this plot since the number of bonds split per minute is constant during the early part of the reaction, we arrive at the bizarre answer of 1.5 strands. This sort of puts us halfway between Watson and Crick, I suspect.

Now this immediately raises the question of what is meant by 1.5 strands, i.e., whether some molecules have two strands and others only one or, what some of us would like it to mean, that on the average each molecule has 1.5 strands. This, in effect, would be equivalent to the suggestion Dekker and I made a year ago about the probable existence of breaks in the backbone structure of DNA. Or the 1.5 may mean any

one of a number of other, perhaps just as likely, alternatives. If the data are analyzed very carefully, as we think we have done at Berkeley, we should be able to calculate the number of preexisting breaks in the backbone. In doing this we assumed that no additional flexibility is introduced by each enzymic attack. To allow for this would compound our difficulties — already plentiful. The result of the calculation shows that if there were only 7–10 preexisting breaks in the macromolecule of molecular weight 6×10^6 , our kinetics would have given us 1.5 strands instead of 2. This, of course, is far fewer interruptions than we had suggested earlier. Our speculation was based primarily on end-group determinations inferred by the people who were analyzing the titration curves of DNA. I now learn via the “grapevine” of private communication that these interpretations of the titration data are in error, and the region of the curve that had been attributed to secondary phosphoryl dissociations, i.e., originating from monoesterified phosphate groups, is better accounted for by amino groups. If this turns out to be correct, then the major foundation for our proposal of interruptions would vanish. The number of breaks determined by this enzymic approach is indeed quite low. It should be stressed that this method, which we suggested some time ago and which we have just discussed, is not without tremendous difficulties. We do not know about enzyme specificity. We do not really measure our viscosity at sufficiently low shear gradient. And we have real problems because of the tremendous polydispersity about which you will hear more in this symposium. These difficulties, it seems to us, are not much lessened by the use of light scattering.

This covers one aspect of Doty’s talk and I would like now to spend a few minutes on the denaturation of DNA.

Figure 1 shows the effect of acid. We agree in the main with the results obtained by Doty and his collaborators a few years ago. This figure illustrates the application of the ultracentrifuge at very low concentrations of DNA. We start with the idea that a viscosity decrease upon change in the

environment of the DNA does not necessarily mean a decrease in molecular weight; but a viscosity decrease coupled with an alteration in the ultracentrifuge pattern will differentiate between a shrinkage or collapse of the macromolecules into a more compact shape as against a large decrease in molecular weight. In order to work at concentrations low enough to minimize difficulties due to intermolecular interaction, we

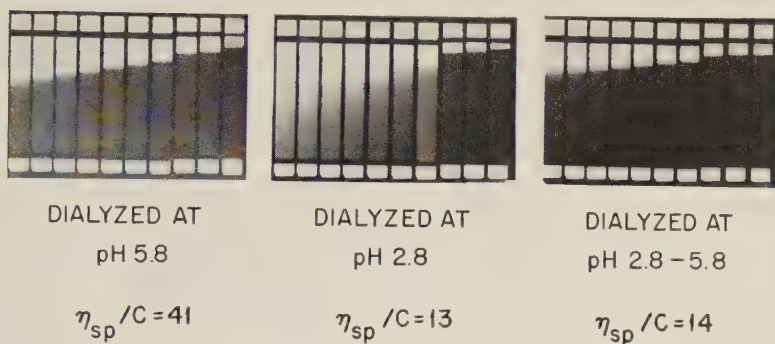


Fig. 1 Ultracentrifuge patterns showing the effect of acid on DNA. In each set of photographs the pattern on the extreme right was obtained at about 5000 rpm, the next at 10,000 rpm, and the third from the right at 30,000 rpm. The remaining photographs, from right to left are obtained at 59,780 rpm at intervals of two minutes. The group of photographs on the left are the patterns for the control DNA solution. In the middle are the photographs for the solution dialyzed to pH 2.8, and on the right are the patterns for the same solution after the pH is again raised to 5.8 by dialysis. The values of the reduced viscosities are beneath the photographs. All physical measurements were performed in 0.2 *M* NaCl, although the exposure to acid was in 0.01 *M* NaCl.

employ ultraviolet absorption optics to follow the sedimentation of the DNA. In these photographs the black region corresponds to the ultraviolet absorbing region and the white regions correspond to the supernatant region now free of DNA. In an attempt to repeat Doty's experiment we started at pH 5.8, dialyzed the solution against a solvent at pH 2.8, and then finally dialyzed the solution again to pH 5.8. As seen from the figure, the viscosity decreases upon lowering of the pH but the sedimentation coefficient increases tremendously. In fact, some of the material is clearly aggregated

and sediments during the acceleration of the rotor. When the pH is returned to 5.8, we find a large reduction in sedimentation coefficient to a value less than that of the original. The reduced viscosity has not returned to the original value, indicating that so many hydrogen bonds had been broken that rehealing to form the original structure was not achieved. Thus the molecular weight has been reduced but not very greatly; and we would agree that the first effect of acid is to cause the molecules to coil up into a more compact configuration. Probably the value of the final pH , the ionic strength, and the length of time of treatment with acid are all important; we note that Doty has now also observed degradation in mildly acid conditions.

I think the effect of heat is somewhat different and this is illustrated very briefly in figure 2, where the results at different ionic strengths are presented. The control solution is used first, and the polydispersity of the DNA is considerable. These boundaries are very broad; I shall have more to say about this in my closing remarks. If this material is heated for 15 minutes at $100^{\circ}C.$, the viscosity decrease is very marked. If the heating is performed in $0.2 M$ NaCl, the sedimentation rate hardly changes at all. Sometimes there is a slight decrease and at other times there may be a slight increase, depending on prior treatment of the DNA. In the absence of salt, however, not only is the viscosity decreased tremendously but the sedimentation coefficient also is decreased a great deal upon heating. This means a rather large decrease in molecular weight. Schumaker, at Berkeley, is going into this matter in great detail and our tentative picture includes three different stages in this process. The first would be what Doty has already mentioned — the denaturation or collapse of the molecule due to rupture of the hydrogen bonds. The second involves a splitting of the macromolecule into smaller pieces; and the third, and this is the worst part of the situation if one wishes to disentangle the three effects, is the liberation of free purines. The last effect probably labilizes phosphoester bonds, causing further de-

gradation. Whether there is a rupture of covalent bonds (phosphodiester bonds) during the early stages (the second stage before purines are liberated) is something that, of course, is crucial to interpretation of the results. We have attempted to find the ionizable phosphate groups that would have been created by hydrolysis of a phosphoester bond, using the buffer indicator technique employed by Cavalieri a few years ago. Thus far we have been unable to find the requisite number of liberated hydrogen ions and our tentative conclusion of a few years ago is still unchanged. I want to emphasize that we have not yet proved to our satisfaction that there is degradation without rupture of covalent bonds.

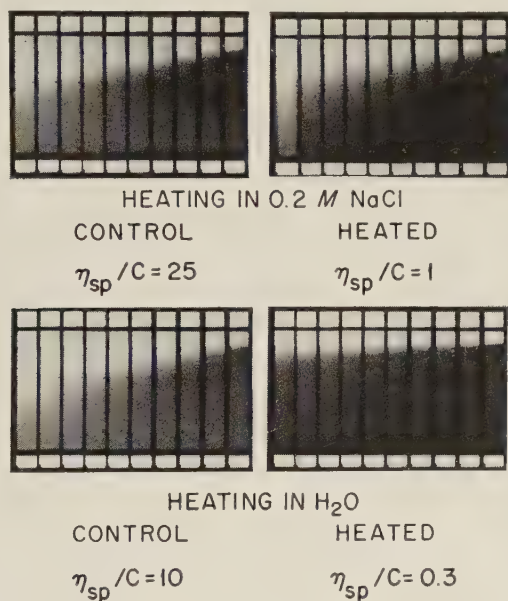


Fig. 2 Ultracentrifuge patterns showing the effect of heat on DNA. In each set of patterns the pictures read from right to left at intervals of two minutes at 59,780 rpm. All physical measurements were performed in 0.2 *M* NaCl. The control DNA solutions on the left were obtained by dissolving the DNA in 0.2 *M* NaCl, upper, and distilled water, lower. On the right are shown the patterns obtained after heating those solutions for 15 minutes at 100°C. The partial denaturation of DNA by dissolution in distilled water is illustrated by a comparison of the polydispersity exhibited by the patterns and the viscosities of the two control solutions.

It seems to me, at least, that the solution to the question about the presence or absence of interruptions rests ultimately in the development of good end-group methods such as the use of a pure phosphomonoesterase.

Now I should like to talk about the last aspect of our work in relation to Doty's paper. This is concerned with the molecular weight and molecular weight distribution of DNA, about which very little is known. We have converted our ultracentrifuge patterns into plots of the weight distribution of sedimentation coefficients as a function of the sedimentation coefficient, much like the type of plot employed by J. W. Williams and his group at Wisconsin. In the calculation of these distribution curves we assume that the ultracentrifuge measurements at a few thousandths of 1% are free of the well-known anomalies that ordinarily plague ultracentrifugal analysis of elongated macromolecules; i.e., we are making our measurements essentially at infinite dilution. With some labor we can obtain distribution curves that are independent of the time of sedimentation even though the boundary becomes almost 1 cm wide during the run. The curves are also independent of the speed of the rotor. These are indications that the centrifuge is behaving ideally and that convective disturbances are not causing any serious distortion of the boundaries. This is a serious problem when solutions are analyzed at a concentration of only 0.001%. To the best of our knowledge we are safe on this score, and we can assume that the distribution curves are representative of the sedimentation coefficients within a given sample of DNA. Next we must worry a great deal about the relation of sedimentation coefficient to molecular weight for such a material. I am inclined to agree with Doty that the intrinsic viscosity is proportional to about the first power of the molecular weight and that the sedimentation coefficient is proportional to the molecular weight raised to the one-third power. With these assumptions, we can make a fair preliminary estimate of the ratio of weight to number average molecular weight, which turns out to be in the neighborhood of

3:1. If further work substantiates this, then perhaps we ought to worry a little more about the extrapolation of the light-scattering data to zero degrees to give the weight average molecular weight.

In disagreement with Doty's remark about the similarity of different DNA preparations, I would like to talk about some DNA preparations that we recently examined, since we feel that all DNA's are not necessarily the same. Figure 3 shows the distribution curve for a preparation of calf thymus DNA and also for a DNA isolated from bacteriophage by Dr. S. S.

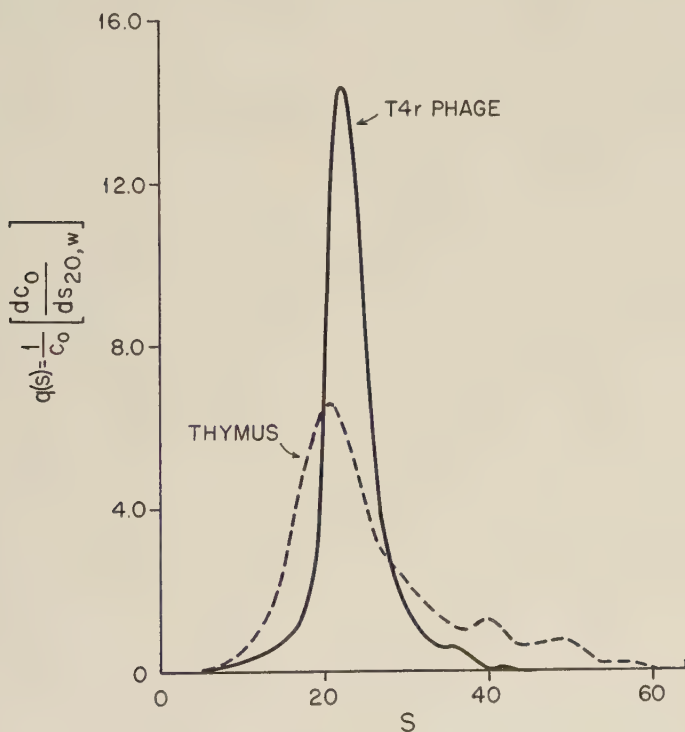


Fig. 3 The distribution of sedimentation coefficients of two preparations of DNA measured at a concentration of 0.003% by ultraviolet absorption optics on the ultracentrifuge. The dotted curve was obtained with the DNA from calf thymus and the solid curve from a DNA isolated from T4r bacteriophage. The area under the curve between any two values of the sedimentation coefficient (on the abscissa) gives the weight fraction of the material having sedimentation coefficients between the selected values.

Cohen. Note that for phage DNA at comparable concentrations, the distribution of sedimentation rates is much narrower, the average sedimentation coefficient is higher, and the viscosity is much higher. Instead of $69 \text{ (g/100 ml)}^{-1}$ for the intrinsic viscosity, we obtain values of $95 \text{ (g/100 ml)}^{-1}$ or higher at zero shear gradient. This material is so large that the ultracentrifuge patterns are still artificially sharp and the sedimentation coefficients measured even at concentrations as low as 0.003% are lower than the infinite dilution values. As a matter of fact, we shall have to improve our absorption optical system somewhat to make it sensitive enough for work at even greater dilutions.

Now I would like to raise one final point not discussed by Doty. If DNA does show this tremendous polydispersity in sedimentation rate, we must ask whether this necessarily means a polydispersity in molecular weight. In principle, there could be a polydispersity with respect to shape that would account for the ultracentrifuge patterns. It should be possible to differentiate between heterogeneity in molecular weight versus shape, and we are now working on this problem, i.e., whether the more slowly sedimenting material has the lower sedimentation coefficients because it is smaller or because the macromolecules are more elongated and therefore experience greater frictional resistance to motion in a centrifugal field. If it is the latter and the solution could be fractionated to yield the slowly sedimenting molecules, this material should have a higher intrinsic viscosity than the bulk of the DNA. In an attempt to answer this question, we have performed some crude fractionation experiments by zone centrifugation. The existing techniques for zone centrifugation are still somewhat inadequate, but we have performed these centrifugal fractionations in sucrose gradients that minimize convective disturbances. From several preliminary experiments we have concluded that the trailing material in the ultracentrifuge is certainly smaller in molecular weight since it is less viscous than the leading material. However, it looks at present as if the intrinsic viscosity of the trailing

material is not sufficiently less than that of the leading material for us to state that the polydispersity is exclusively in molecular weights. This can be stated in an alternative fashion. The exponent, α , relating viscosity to molecular weight does not appear to be a constant, independent of molecular weight, but rather it decreases somewhat as the molecular weight increases, suggesting that the larger molecules are more folded. These results are only preliminary, and it is clear that we must learn more about the molecular weight distribution of DNA preparations. Then we would be in a better position to consider the configuration of the macromolecules in solution.

In conclusion, it might be well to restate what C. A. Dekker and I tried to emphasize in our review a few years ago, that is, that the studies of DNA that you have heard about in this symposium have been performed on many preparations isolated by different procedures. The macromolecules have been subjected to varying influences during the isolation. Some preparations were more denatured than others, some contained more protein than others, and some had more breaks or even may have been more degraded than others owing, presumably, to inadequate inhibition of deoxyribonuclease during the isolation and purification of the DNA. This may provide an explanation for the discordant results of different investigators. It would also highlight the need for working with DNA that possesses some biological activity.

MACROMOLECULAR FABRICS AND PATTERNS

ROUND-TABLE DISCUSSION

BACKGROUND

PAUL WEISS

Rockefeller Institute for Medical Research, New York City

The presentation this evening marks the opening event of a series of conferences that will extend through the summer of this year and is designated as the "Developmental Biology Conference Series 1956." It is being held under the sponsorship of the Biology Council of the National Academy of Sciences and will bring a large number of American and foreign scientists together in interdisciplinary groupings for a discussion of some of the most fundamental problems in the field of development and growth, in which close and concerted cooperation between the biological and physical sciences is urgently needed. These two groups approach the phenomena of life from opposite directions. The biologist, who, by observational and grossly analytical procedures, strives to resolve the organism and its functions into smaller and smaller constituent units and their interactions, hopes eventually to link up with the physical scientist who tries to project his knowledge of the molecular events in nonliving systems upward into the domain of life processes. Their efforts are comparable to the building of a tunnel from both ends, in which the twain will not meet unless each keeps his blind shaft oriented toward the other. To recheck this orientation and, if needed, to enhance its straightness, is one of the purposes of this conference series, with special regard to the problems of development and growth.

The biologist, working downward from his end, frequently does not drill deep enough to present the physical scientist

with problems sufficiently concrete and explicit to be picked up from the other end for further analysis and resolution into molecular terms. Reciprocally, those coming from the molecular dimensions are frequently unfamiliar with, or unmindful of, the real nature and substance of the complex biological phenomena that they are aiming to approach, and hence get diverted into directions that lead away from, rather than toward, the common goal of a breakthrough. In the meantime, both groups take comfort from the use of symbolic verbal substitutes for knowledge as bridges over what still is an enormous gap and, more often than not, accept these verbal bridges as if they were permanent fixtures of their conceptual structure rather than merely temporary expedients to be replaced by factual knowledge, the sooner the better.

It is in the service of this common goal that I consider myself privileged in being allowed to present to you some recent observations that do seem to bring the knowledge of molecular events and that of their behavior in the organism one step closer.

The other conferences of the series that are to follow will carry this theme further with regard to problems such as cell differentiation, wound healing, hormone actions, cell division, and growth control. It is hoped that those participating in these exercises will return to their workbenches on the molecular, organismic, or intermediate levels, as the case may be, with a more realistic outlook on what the other workshops in related fields are doing, hence with broader vision and new beacons for the self-direction of their own particular research.

MACROMOLECULAR FABRICS IN BIOLOGICAL SYSTEMS; THEIR STRUCTURAL AND PHYSIOLOGICAL SIGNIFICANCE¹

FRANCIS O. SCHMITT

Biology Department, Massachusetts Institute of Technology, Cambridge

TWO FIGURES

Examination by high resolution electron microscopy of various types of natural substances (e.g., fibrous proteins, lipids, nucleic acids, and polysaccharides) as well as various components of tissues and cells in thin sections has already revealed a wide variety of patterns of structure. Some of these structures may have great cytological and biochemical significance, being more or less self-contained partial systems within the protoplasmic microcosmos and having certain specialized functions that may or may not now be fully understood. Mitochondria represent such morphologically distinctive structures with highly specialized roles in the biochemistry and physiology of the cell. The Golgi system and also the lamellar system of the cytoplasm, which when coupled with RNA-rich granules represent the basophilic component of the cytoplasm, may be included among such systems as may the nucleolus of the nucleus and, of course, the chromosomes themselves. Other structures that have been observed may have no general significance but may pertain only to a particular cell under very special conditions.

In the early phases of such morphological investigations, particularly after the introduction of a powerful new tool

¹These studies were aided by a research grant (B-24) from the National Institute of Neurological Diseases and Blindness, of the National Institutes of Health, U. S. Public Health Service; and by a grant from the Trustees under the wills of Charles A. King and Marjorie King.

such as the electron microscope with its enormous resolving power, it is hardly possible to do more than describe the various types of structures seen and to attempt to classify and catalog them in a fashion that may not only be useful for descriptive purposes but that will hopefully not be misleading to the biochemist and physiologist trying to understand the function of the structures. Unfortunately, morphological terms frequently fail to emphasize that aspect of the structure that is significant biochemically or even biologically but emphasize an aspect that is either inconsequential or actually misleading.

After the first purely descriptive phase, such as that of electron microscopy in which we are now working, will follow a period in which it will become possible to interpret the previously observed patterns and fabrics in terms of the molecules and macromolecules of which they are composed. It is at this level of organization that explanations of physiological processes become most meaningful. Only after the "molecular machinery" has become known will it be possible to understand how the chemical energy from the metabolic pool gets coupled with the protoplasmic machinery to make possible the physiological processes characteristic of the tissue.

The newer discoveries in tissue ultrastructure confirm the idea that, with increasing complexity of organization, new chemical and physiological properties emerge. It is already becoming obvious that certain structural idioms characterize plant and animal cells generally and probably meet certain basic biological needs. In this paper we shall consider two of these structural idioms—the linear, fibrous arrays and the lamellar, membranous systems. The former is important in mechanisms specialized for mechanical purposes (such as the fibers of connective tissue, muscle, and the dividing cell) and for a specific linear sorting out of chemical groups (chromosomes). The membranous systems provide the enormous surface so characteristic of protoplasm: the "floor

space'' upon which to assemble, in special array, the various molecular machines (enzymes and other biocatalyzers) needed in the chemical metabolism of the cell.

It now appears that to solve the needs of the first type, the fibrous systems, there were developed highly elongated building units that possess not only the necessary properties of anisotropy but also a high degree of specificity of chemical structure and reactivity. This was apparently accomplished by the hooking up, in linear array, of relatively small protein molecules made by the cell. The process of lineation leads not only to the development of long, thin macromolecules with new mechanical properties but also to an array of chemical groupings and electric charges that are also of a novel and emergent kind as compared with the smaller parent protein. The helically coiled polypeptide chain construction of the component smaller protein molecules provides an important clue to the origin of such emergent properties. In this paper we shall describe and evaluate some of the clues that electron microscopy has now provided about the structural and chemical properties of the long, thin macromolecules and their fibrous aggregates.

The large surfaces needed in cellular economy have been provided for by the widespread use of lipid and lipid-protein films. These consist of bimolecular leaflets of mixed lipids bonded more or less firmly to protein and other polar constituents at the aqueous interfaces. The relatively long hydrocarbon chains of the lipid molecules exert strong van der Waals interaction, providing strong attractive forces between these portions of the molecules; this together with the mutual interaction of the polar groups, at the aqueous interfaces, with each other and with protein components provides the two-dimensional, membranous structure that characterizes the limiting envelopes of the cell, the nucleus and various cytoplasmic particulates, and the elaborate membranous system developed in the cytoplasm of certain metabolically active cells. We shall examine certain structural patterns developed

by cellular membranes and shall inquire briefly into the nature of the interaction of such surfaces with each other.

FIBROUS FABRICS

High resolution electron microscope studies have revealed certain characteristic types of fibrous structures in cells and interstitial tissues: (1) smooth-contoured filaments and fibrils having constant width and indefinite length (such as the cellulose fibrils of the plant cell wall); (2) tactoidal structures of elongated but irregular shape and no obvious axial or transverse periodicity; and (3) most important for present purposes, fibrils that manifest a high degree of structural regularity, frequently in the form of a regular cross-banding with a characteristic axial repeat pattern. Among the fibrous types having a regular axial periodicity (and frequently a considerable amount of intraperiod fine structure) are collagen, protozoan trichocysts, paramyosin, fibrin, striated-muscle, and light meromyosin.

Much careful study has been devoted to the details of axial fine structure of the fibrous protein in the attempt to correlate the structure with chemical and crystallographic data. Thus far this has not met with great success except in a very general way.

Very valuable also in the analysis of such fibrous organization has been the property (shown particularly well in collagen) of the native fibrils to disperse or "dissolve" in an appropriate chemical medium and then, by subsequent alteration of the composition of the medium, to precipitate out as highly structured fibrous arrays. The reconstituted fibrils may have either the native type of fine structure or certain novel types that have nevertheless proved very valuable as aids in the interpretation of underlying macromolecular structure.

Basic to the investigation of the detailed structure of such fibrous systems is the isolation of the molecular or macromolecular units and their characterization by methods of

physical chemistry and crystallography. Such information is necessary before substantial progress can be expected in the investigation of the fundamental problem or the nature of the forces that cause these highly organized macromolecular arrays to aggregate and disaggregate as they do when placed in certain chemical environments. Only the barest beginning has been made toward a theoretical approach to this problem of the specificity of macromolecular interaction. Meanwhile it is urgent that biologists assemble experimental evidence about the various types of macromolecular systems existing in tissues and that can be isolated and subjected to the types of analytical procedures that may eventually provide the evidence needed for a definitive physical-chemical analysis.

At the present time relatively few fibrous proteins capable of such reversible interaction have been carefully studied. One of these is the fibrous protein of the connective tissue, collagen. Since it is the protein with which I have had most experience, it will be used to illustrate principles that may prove characteristic of many other fibrous systems.

Collagen: A prototype of fibrous proteins

In the last decade or so great interest has been shown in the analysis of the properties of collagen by physical chemists, crystallographers, electron microscopists, biochemists, and biologists. As a result this protein, which was long ago assigned to a position of prominence among fibrous proteins for purposes of classification by Astbury ('40), now has become something of a prototype among fibrous proteins. We shall therefore use this protein to illustrate the manner in which the various techniques may be applied in approaching nearer to a solution of the properties of fibrous systems.

We may consider first the axial periodicity and the pattern of banding as seen in the electron microscope. Although serving as a sort of "molecular finger print" of collagen the pattern is by no means uniform even in purified material. As few as two and as many as thirteen intraperiod bands

(fig. 1) have been observed (see Schmitt et al., '55a; Nemetschek et al., '55). What is observed in a given specimen must depend upon the perfection of matching up of discontinuities along neighboring chains. It is therefore difficult, on the basis of a study alone of the fine structure of native

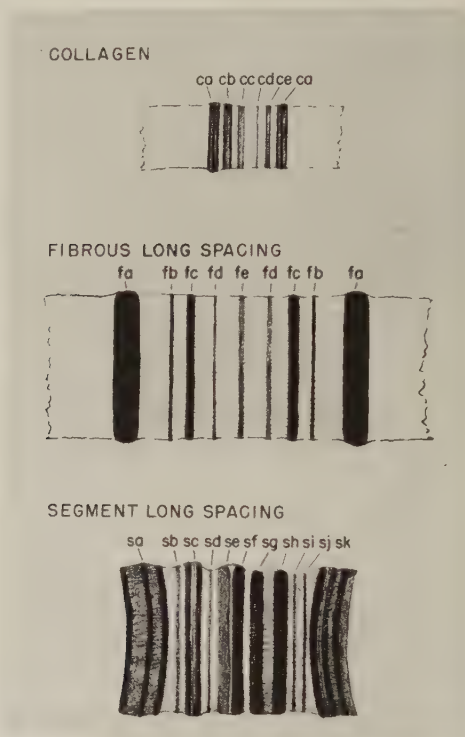


Fig. 1 Diagrammatic illustrations of intraperiod fine structure of native collagen fibrils, fibrous long spacing, and segment long spacing. For details see Schmitt, Gross, and Hightberger ('55a).

collagen fibrils to interpret this structure in terms of specific entities such as collagen molecules or specific amino acid residues.

Important, however, in the interpretation of the significance of the band pattern is the general concept of the physical basis of bands (the regions that are slightly thicker and stain more intensely) and interbands. From the characteristic

fraying of fibrils, it was obvious from the first that the protein chains run preponderantly longitudinally; the smallest such fibrous unit was called the protofibril by Schmitt, Hall, and Jakus ('42). Banding must involve a periodic variation in the interaction of these units and Bear ('52) suggested that the bands and interbands represent regions of relative disorder, and order, respectively, caused by interaction between the side chains of adjacent main chains or protofibrils. Where the side chains are large and bulky it is impossible for them to pack perfectly and therefore they form regions of relative disorder (bands). Because of the differing chemical nature of the end groups of the side chains as well as differences of packing there are corresponding differences in solvation and of combination with stains in the band and interband regions. For present purposes we shall adopt Bear's explanation of band and interband structure.

An extremely valuable technique by which electron microscopic evidence may be obtained about the units of fiber structure is that of dissolving the fibrous material in an appropriate solution, and then reconstituting the material by alteration of *pH* or ionic strength. By such experiments a new "fibrous long-spacing" modification of collagen was discovered (Highberger et al., '50) and later a "segment long-spacing" form (Schmitt et al., '53). The structure of these long-spacing forms is shown in figure 1. These led to the deduction that the kinetic unit of collagen is a long (2000–3000 Å), thin macromolecule to which was given the name "tropocollagen." The long-spacing types are in general not formed in solutions of relatively high ionic strength and this probably explains why they have not thus far been observed in tissues.

The band patterns and axial periods not only of the native but also of the various reconstituted forms of collagen, including the long-spacing types, were then interpretable in terms of tropocollagen macromolecules polarized in the same (parallel) or opposite (antiparallel) directions, in register with respect to macromolecular ends or staggered by specific

fractions of macromolecular lengths. In this interpretation the protofibril is considered to be a polymerization or end-to-end linking of tropocollagen macromolecules.

It should be emphasized that, viewed in this manner, neither the tropocollagen macromolecule nor the protofibril is banded. Only when these units aggregate laterally, resulting in specific side chain interaction, does banding result.

Essential in the analysis is the development of methods by which the structural units or macromolecules may be isolated in monodisperse form and characterized by application of the various techniques of physical chemistry. In the case of collagen this was accomplished by Boedtker and Doty ('55, '56) who showed that, in citrate buffer, the tropocollagen macromolecules have dimensions of 14 by 2900 Å.

Direct visualization of the macromolecular units by electron microscopy is highly desirable not only as a check on the conclusions arrived at by physicochemical and crystallographic methods but also because of information which it alone can give. Up to now, this has been impossible because the structure of the substrates on which the macromolecules were deposited was as great as or greater than that of the macromolecules to be examined. However, by a new method, involving the deposition of the dissolved particles on a surface of freshly cleaved mica followed by shadowing with platinum, Hall ('56) succeeded in resolving various kinds of fibrous structures 15–20 Å wide. The protofibrils having indefinite length and the individual tropocollagen macromolecules with widths of about 15 Å and lengths averaging about 2200 Å were clearly resolved.

Meanwhile it was shown by X-ray studies (see particularly Rich and Crick, '55, and the most recent in this series, Bear, '56) that the collagen macromolecule contains three polypeptide chains helically coiled and bonded to each other chiefly by hydrogen bonds. From denaturation studies, Boedtker and Doty ('55, '56) concluded that the three chains may have different lengths and may not completely overlap in the native tropocollagen. The possibility that the chemical

composition of the three chains may differ, particularly in their hydroxyproline content, has also been claimed. It has also been suggested (Highberger and Schmitt, unpublished) that the length of the tropocollagen macromolecule in solution may be susceptible of considerable variation because the amount of overlapping of the three intraparticle chains may be subject to considerable variation. Such a hypothesis would assume a considerable lability of the hydrogen bonding of the chains to one another.

Under certain conditions the tropocollagen macromolecule is thus not a uniform building block of constant dimensions and properties but a particle composed of three chains that may vary their positioning with respect to one another. The pattern of side chains, hence the band patterns of the fibrils formed from the macromolecules, may also vary depending on conditions. The type of structure formed depends not only on the built-in structure of the macromolecules but also very sensitively on the chemical environment that determines the ionization of side chains, degree to which prosthetic compounds may combine with the collagen, and so on. Thus the long-spacing structures appear to require for their formation the presence of small amounts of certain types of molecules such as α -1 acid glycoprotein, chondroitin sulfate, hyaluronate, heparin, DNA, RNA, and ATP. It seems probable that these substances exert their effect by combining with specific side chains of the collagen, thereby preventing (or causing) certain types of aggregation of the macromolecules. Thus when ATP is reacted with tropocollagen in acid solution, it probably combines primarily with the lysine side chains and prevents the tropocollagen macromolecules from combining with each other in either staggered or antiparallel array; as a result, neither the 640 Å type of band pattern nor the fibrous long-spacing type can be produced. Rather, the macromolecules then combine in a form that produces the segment long-spacing type in which the tropocollagen particles are thought of as being polarized all in the same direction (parallel array) and with ends approximately in register.

Before the discussion of the origin of the band patterns and axial periodicities in protein fibrils is concluded, a matter of importance in the application of this type of analysis to other biological macromolecular systems should be made very clear. If this point is missed, only confusion will result when the biologist tries to understand, for example, how information about interaction properties of macromolecules derived from electron microscope studies of a protein such as collagen can throw light on the macromolecular properties of chromosomes. The point is that merely because a particular fibrous type shows no bands under the electron microscope is no proof that its fibrils are not composed of elongated macromolecules having highly specific interaction properties. If the fibrous type in question does not happen to possess side chains of such size and chemical properties as to produce disordered regions on interaction with neighboring molecules no bands will be seen in the electron microscope. For example, DNA has no long side chains and the narrow (100–200 Å) fibrils formed by linear and lateral aggregation of DNA macromolecules appear unbanded in the electron microscope. Yet there is every reason to believe that DNA macromolecules, as in chromosomes, interact with each other and with other types of macromolecules in highly specific fashion (Schmitt, '56a,b). For studies of such interaction patterns, the lessons learned from investigations of systems like collagen, whose band patterns do greatly facilitate interpretation of macromolecular interaction properties, should be fully utilized.

Significance of macromolecular structure and interaction patterns in physiological function

The mechanical and other properties of the macromolecular aggregates that form the machinery of the cell are determined by the internal structure of the macromolecules and by the ways in which they aggregate to form the various fabrics such as occur in cells and tissues. However, if this were the whole story the machinery would be, so to speak, a very lifeless

one indeed. But when specific types of substances in the chemical environment (particularly substances such as ATP, which have available potential energy within their molecules) interact with the macromolecular machinery, then it becomes possible for these fibrous arrays to interact in vigorous and characteristic fashion. This is function at the molecular level. It may be illustrated by a few examples taken from research currently being stressed.

Connective tissue. Being essentially mechanical in function the collagen fibers of connective tissue manifest high tensile strength (50–100 kg/mm²). This is made possible by the particular fashion in which the macromolecules interact to form the highly organized cross-striated collagen fibrils (strong lateral interaction integrated over a large fraction of the surfaces of the overlapping macromolecules). Such collagen fibers are probably fairly inert mechanical structures most of the time.

The collagenous systems in tissues may, however, also present a very dynamic aspect of considerable biological and medical significance. The steps in the synthesis of the individual polypeptide chains in tropocollagen, their assemblage into macromolecules, and the interaction of the tropocollagen to form native collagen fibrils must be under enzymic control and must be very dynamic processes. This may be particularly significant in processes of aging and in pathological conditions where collagen is actively laid down (or removed) in adult tissue. It is also probable that the interaction of the collagen fibrils of adult connective tissue may be influenced by hormones and other organic materials. This possibility is being studied in *in vitro* systems by optical methods in our laboratory at the present time.

Muscle and contractility. Electron microscope studies in many laboratories have demonstrated that in striated muscle (and probably in any type of muscle), we are dealing with a parallel array of at least two types of fibrous proteins, myosin and actin, and that changes in length and tension of the myofibril depend on a characteristic pattern of interaction

between these two fibrous proteins when the chemical environment is caused suddenly to change by the passage of the excitatory impulse. The specific manner in which this interaction occurs has not yet been clearly demonstrated (see Hodge, '55; Hanson and Huxley, '55; Perry, '56). Spiro ('56) proposes that the interaction is essentially a coiling of one constituent (actin) about another constituent (myosin) causing the constituent that forms the continuous array (actin) to be supercoiled and thus causing the entire fibril to contract. Whatever the specific details of the process may prove to be it seems safe to assume that contraction will involve the principle that when molecules of ATP, or other source of chemical energy, combine with one or both of the fibrous proteins, chemical sites are thereby occupied, which strongly affects the interaction of the two proteins.

Blood clotting (fibrinogen-fibrin transformation). Blood clotting is an excellent example of a very important physiological process that involves the formation of insoluble fibers of fibrin (which, after retraction, occlude the blood vessels) from soluble fibrinogen molecules and that is under the control of a nicely balanced system of activators and inhibitors. Activation of the clotting process causes the cleavage of a small portion (fibrinopeptide) of the fibrinogen molecule. This change of configuration causes the fibrinogen molecules to aggregate rapidly and form intermediate polymers that subsequently unite as the fibrin clot (see Ferry et al., '54; Siegel et al., '53). The fibrils so formed appear banded in the electron microscope, the axial period being about 235 Å (Hawn and Porter, '47; Hall, '49). According to Ferry et al. ('54), fibrinogen molecules aggregate as intermediate polymers by forming double rows of molecules in staggered array. It seems probable that the bands and interbands as seen in the electron microscope result from this type of staggered lining up of the fibrinogen molecules, in analogy to the collagen case.

Chromosomes, genes, and macromolecular systems. According to recent physicochemical analyses the DNA in chromosomes occurs as very long, thin (30,000–40,000 Å by 20 Å) macromolecules (see Doty, '55). In the giant salivary gland chromosomes of dipteran insects the DNA interacts in a highly specific fashion with one or more fibrous proteins, histones and protamines, to produce an *aperiodic* banded structure readily visible in the light microscope. Gene maps have been constructed that localize particular genes with respect to particular bands. Extraction and solubility studies of chromosomes (Mazia, '54) indicate that the bonding between macromolecules is very weak (chiefly hydrogen bonds and electrostatic bonds). It was suggested (Schmitt, '56a,b) that, although highly localized regions within the DNA macromolecule may constitute genes (as deduced from recent studies of plant and animal viruses), it is also possible that interaction between macromolecules (between DNA and DNA, or DNA and protein) may activate, inactivate, or protect particular chemically active groups on adjacent macromolecules. Such a macromolecular interaction theory is consistent with current concepts in genetics (see Goldschmidt, '55; Lewis and Schmitt, '56). For more detailed discussion of this theory see Schmitt ('56a,b).

INTERACTION CHARACTERISTICS OF SURFACE FILMS AND MEMBRANOUS SYSTEMS; BIOLOGICAL SIGNIFICANCE

Because of the great physiological and biochemical significance of the plasma membrane, the nuclear membrane and the various interfacial films in protoplasm, much effort has been made over many years to determine their chemical constituents and their molecular architecture. This has involved both the direct analysis by biophysical and biochemical methods and indirect deduction from a study of permeability and other physiological properties. Studies of the posthemolytic membranous residues (ghosts) of mammalian erythrocytes have been very fruitful (see Ponder, '55).

From such studies, the conclusion has emerged that such cellular films consist essentially of a bimolecular leaflet of mixed lipid molecules oriented with paraffin chains normal to the plane of the film and conjugated more or less firmly at one or at both aqueous interfaces with a thin, possibly macromolecular, layer of protein or other polar compounds. This has in general been confirmed by electron microscope examination of such films in thin sections. However, as yet such studies have not yielded as definitive evidence as one might have hoped for, primarily because of the difficulty of interpreting the fixation artifacts, such as dense single- or double-edged structures, seen in osmicated tissue.

The lipid molecules that are present in such films are particularly well suited for the purpose because they possess one or two long hydrocarbon chains and a strongly polar, frequently ionized, polar end. The strong van der Waals attraction over the long chains serves to maintain the parallel orientation of the chains normal to the surface of the film and the interaction of the polar groups at the aqueous interfaces provides further integration of molecular ends with each other and with the components of the aqueous substrate. The lipid double layers have thicknesses ranging from 40 to 70 Å, depending on the relative concentrations of the different lipid types (Bear et al., '41). The films probably have many properties common to liquid condensed or solid films.

Thus is constructed the fabric that forms the molecular "floor space" for the cellular machinery. When particular enzymes and biocatalysts are mounted upon such a layered flooring, possibly in a particular arrangement with respect to each other and to the film, organelles such as mitochondria, Golgi system, and the cytoplasmic membranous systems (endoplasmic reticulum) are formed. Electron microscopy of these organelles in thin sections has revealed how widespread are the thin osmiophilic membranes, which are presumably composed of lipids and proteins.

Little is known about the structure of the molecular "factory" for the synthesis of the lipids and the formation of the thin films. Mitochondria are involved biochemically in the synthesis (see Kennedy, '55). If one could picture a highly localized region where such elongated bifunctional molecules are being synthesized one might expect that, as they are synthesized, they must of necessity aggregate to form either some kind of micelle or a film. If the biosynthesis occurs at the surface of a thin film, the synthesized lipid molecules might be expected to penetrate, and be incorporated into, the film itself. Thus film material would appear to be capable of increasing its substance. In certain instances, such as that of the Schwann cell in a myelinating fiber, to be discussed in more detail, the plasma membrane may increase by manyfold.

Heavy concentration of lipid-protein layers occurs in certain tissues as in the nerve myelin sheath, retinal rods and cones, and chloroplasts. By a study of the early stages in the morphogenesis and differentiation of such systems, information may be gained about the molecular mechanism of the process. From such a study of chloroplast development, Hodge et al. ('55, '56) suggested that the precursor of the layers is a vesicle-like structure that has as its feature of especial interest a thin, limiting film. Fusion of the limiting envelopes of these vesicles forms the layered system of chloroplasts.

From an electron microscope investigation into the early development of the myelin sheath Geren ('54) made the very important discovery that the lipid-protein layers are not strictly concentric as was previously supposed but are helically wrapped about the axon (see Geren, '56, for a review of these studies). The layers are in fact formed by the infolding of the plasma membrane of the Schwann cell after that cell has surrounded the axon (see fig. 2). At the point of infolding, the outer surfaces of the apposing Schwann cell membrane adhere strongly to each other. As more and more of such double surface membrane is infolded, a spiral structure is

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seen in transverse thin sections. As the process continues the layers coalesce, with expulsion of cytoplasmic material centrifugally and perhaps longitudinally. Thus is formed the compact myelin with its layered structure. Robertson ('55) published an electron micrograph of a transverse section of a myelinated fiber from a mature chameleon that, in one field, shows the outer infolding Schwann cell membrane, the compact

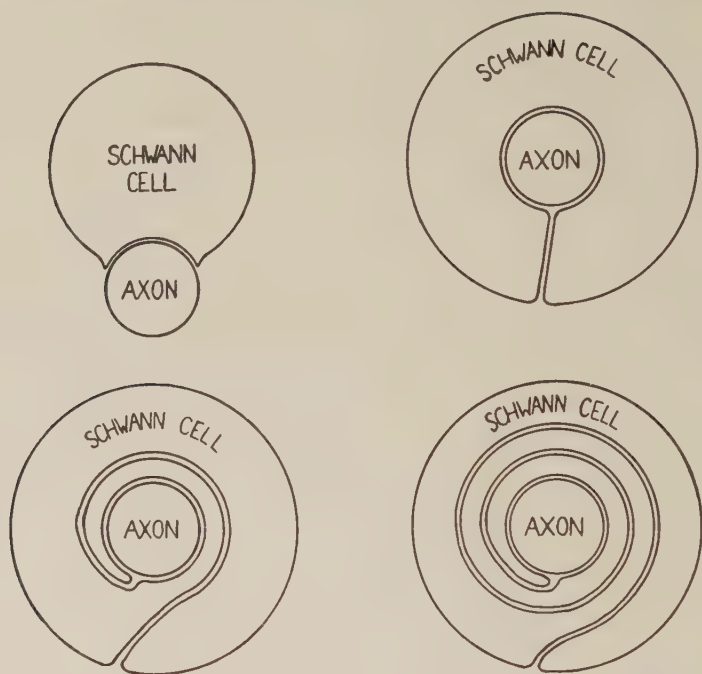


Fig. 2 Origin of myelin layers by infolding of surface membrane of the Schwann cell after the theory of Geren ('54, '55).

myelin layers and the inner Schwann cell membrane applied onto the outer surface of the axolemma. This beautifully confirms the process inferred by Geren from observation of various developmental stages.

This new knowledge about the structure and origin of nerve myelin emphasizes the desirability of extending the X-ray diffraction analysis of myelin structure (Schmitt et

al., '41; and of Finean, '53a,b, '54). From such data we may hope to learn more about the structure of the plasma membrane, which as a single surface membrane, would not be amenable to X-ray analysis but, in the rolled-up form as nerve myelin, yields considerable diffraction data.

The infolding of the Schwann cell membrane is presumably conditioned by the presence of the axon about and around which the Schwann cell applies itself. However, later descriptions of electron micrographs of thin sections of various types of cells, particularly those engaged in active secretion or other forms of metabolic activity, show a similar infolding of the plasma membrane of individual cells. The degree of this infolding is sometimes fantastic (see Pease, '56), indicating that the surface area of the cell had been very greatly increased. A similar infolding of the surface membrane of the thin Schwann cell that encloses the axon of the squid giant fiber has been described by Geren and Schmitt ('55).

In view of the biological significance that obviously attaches to such membrane phenomena, it is important that we attempt to analyze the forces involved in the process. At present little information is available on which to undertake such an analysis. It seems improbable that the adhesion is caused only by an attraction between the polar ends of lipid molecules in the bimolecular leaflets or to a bonding of these layers by ions such as calcium or other multivalent ion. Rather it may be suspected that material, probably chiefly protein in nature and bonded to the outer surface of the lipid leaflets, may play an important role by providing attractive forces between the infolded layers.

In a previous examination of forces between cell surfaces it was shown (Schmitt, '41) that a positively charged protein, such as a histone, may cause strong cohesion of adjacent cells if the cell membranes are negatively charged. A model system consisting of red blood corpuscles, caused to aggregate by the addition of very low concentrations of histone, was described. It was suggested that attractive forces of this kind

might literally "zipper up" the contiguous cell membranes with the expulsion of water, causing adjoining cells to seek to share a maximum of surface in common.

To what extent membrane infolding and the formation of myelin layers may depend upon a protein constituent (such as the neurokeratinogenic protein of the myelin sheath) remains to be seen. The specific aggregation of cells in the formation of morphogenetic fields in embryogenesis has similarly been attributed to specific interaction of substances such as proteins at the surface of the aggregating cells (Schmitt, '55). The degree to which the pattern of interaction of adjacent cells may be influenced by coating the cells with a protein whose molecules are known to be capable of interaction with each other according to various patterns, depending on the chemical environment, is now being studied experimentally in this and other laboratories. Eventually some such link must be forged between specificity of interaction at the molecular and at the cellular levels.

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MACROMOLECULAR FABRICS AND PATTERNS ¹

PAUL WEISS

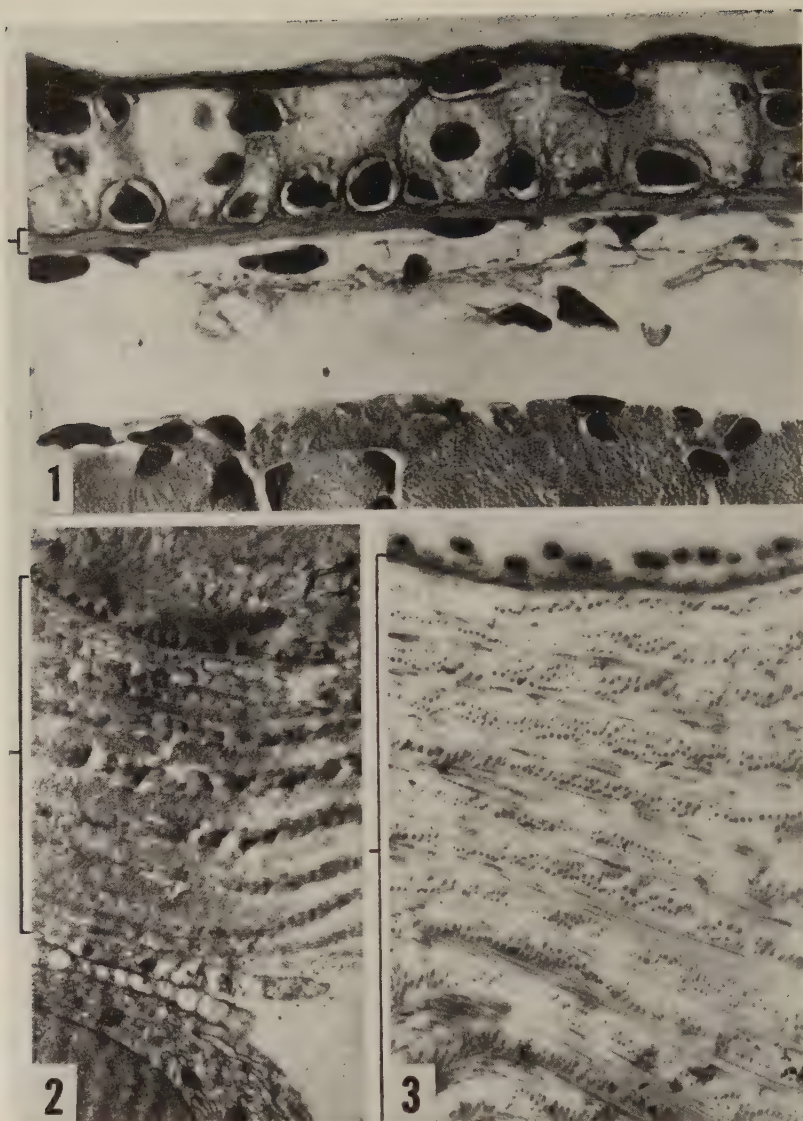
Rockefeller Institute for Medical Research, New York City

FIVE FIGURES

Previous discussions at this symposium have taken you from molecules to their serial combination into filaments and to the grouping of such threads into bundles and skeins. I shall now try to take up from here and carry the process one step further on to the weaving of the threads into fabrics, such as we find in the living tissue. In doing this, I shall stick to a description of the phenomena without indulging in theoretical explanations that, for the time being, are spurious. If any prediction can be ventured about how these phenomena might in the end be mastered conceptually, it is that we may have to postulate some sort of "macrocrystallinity" as a basic property of living systems. But I shall now proceed directly to the facts.

The example, which I am about to illustrate, presents the basic problem in a nutshell. The work was done with the collaboration of Wayne Ferris (Weiss and Ferris, '54a, b). In looking for submicroscopic fabrics of regular geometric design in the organism, I had found, a few years ago, a most spectacular object in the *basement lamella* lining the skin of amphibian larvae. The general disposition of this structure is shown in figure 1 in a microscopic view of a cross section through the skin. The membrane in question, about 4μ wide, can be seen under the smooth top layer of epidermal cells; it separates the epidermis from the underlying loose connective tissue. In ultrathin sections of osmium tetroxide-fixed material under the electronmicroscope, this membrane reveals

¹ Work referred to in this paper was aided in part by a grant from the American Cancer Society (through the Committee on Growth).



Figs. 1-3 Cross sections through basement lamella of skin at three levels of magnification. Thickness of membrane indicated on margin by brackets.

Fig. 1 Microscopic view, showing epidermal cell layer on top, and connective tissue cells beneath basement lamella. $\times 470$.

Fig. 2 Electronmicroscopic view, showing from top to bottom: basal portion of epidermal cell with dark, bobbin-shaped, attachment bodies in lower surface; layered structure of basement lamella; portion of connective tissue cell with endoplasmic reticulum, mitochondria and sector of cell nucleus. $\times 10,000$.

Fig. 3 Electronmicroscopic view at higher magnification ($\times 18,000$), showing the orthogonal sets of fibers.

a sharp lamination, consisting of approximately twenty layers, each 2000–2400 Å in depth (fig. 2). Between the basement lamella proper and the epidermal cell there is a thin intermediate lining, about 600 Å wide, containing a set of spherical granules.

Now, examining the basement lamella under still higher magnification (fig. 3), one notes that it contains a very regular system of cylindrical fibers tentatively identified as collagen. These fibers are about 500 Å in diameter and regularly cross-banded at an axial period of about 520 Å. Within any one layer, all fibers run parallel, with their cross-bands in lateral register. However, the orientation of each successive layer differs from that of the adjoining ones by an angle of 90°. The whole membrane thus is built essentially like plywood with perpendicular alternation of the grain in consecutive plies.

This then, is an example of a supramolecular fabric of a high degree of architectural order. The problem we want to focus on is how such a regular grid — which, incidentally, in this case is extracellular — attains its ordered arrangement. The presentations at this meeting have amply illustrated the elementary properties of the collagen molecules, of their ability to polymerize to long filaments with rhythmic axial patterns as well as to combine laterally and build up bundles in which corresponding fiber segments are laterally aligned. We note that these same elements are utilized in the building of the basement lamella, but what is different between the *in vitro* and *in vivo* condition is the higher degree of organization, of regularity and geometric order, in which the elements are arrayed in the fabric of the body. This difference offered the prospect of leading to some more concrete information as to just what the term “higher organization,” as applied to events within the organism, might connote. We therefore proceeded to trace the steps by which this fabric is formed in the organism, with the following results (see Weiss and Ferris, '56).

When a piece of skin is excised with the underlying patch of basement lamella, the wound heals and the missing patch is gradually restored. We could follow the progress of this restoration by taking samples at various stages and studying them under the electronmicroscope. Shortly after making a wound, the surrounding epidermal cells become detached from their substratum and move in convergent courses over the wound, thus covering the raw area with a continuous layer of cells. Beneath these cells we find a colloidal exudate, which assumes the place of the missing patch of the basement lamella, and right under this are fibroblasts of the connective tissues, destined to produce the new collagen fibers needed for the repair. Without going into the details of this process, let me simply illustrate the results observable toward the end of the first week (fig. 4). We note needle-like new fibers scattered throughout the slab of coagulated exudate, which latter may, in the meantime, of course have undergone chemical transformations that do not express themselves under the electronmicroscope. The young fibers are less than 200 Å wide but show already traces of the mature 500 Å axial periodicity. Their disposition, on the other hand, is quite different from that of a mature membrane: they occupy only about a thousandth of the total space and are arrayed quite at random without any prevailing over-all orientation. These features contrast markedly with the relatively close packing and regular geometric pattern that characterize the old membrane. As will be shown presently, however, the original organization is reconstituted eventually, within a matter of weeks. In fact, at close inspection, figure 4 already reveals the first steps of the ordering process. It will be seen that near the epidermal surface, the young fibers no longer show the confused arrangement dominating the rest of the picture, but display a preferential orientation parallel to the epidermal surface, thus describing the first few laminae.

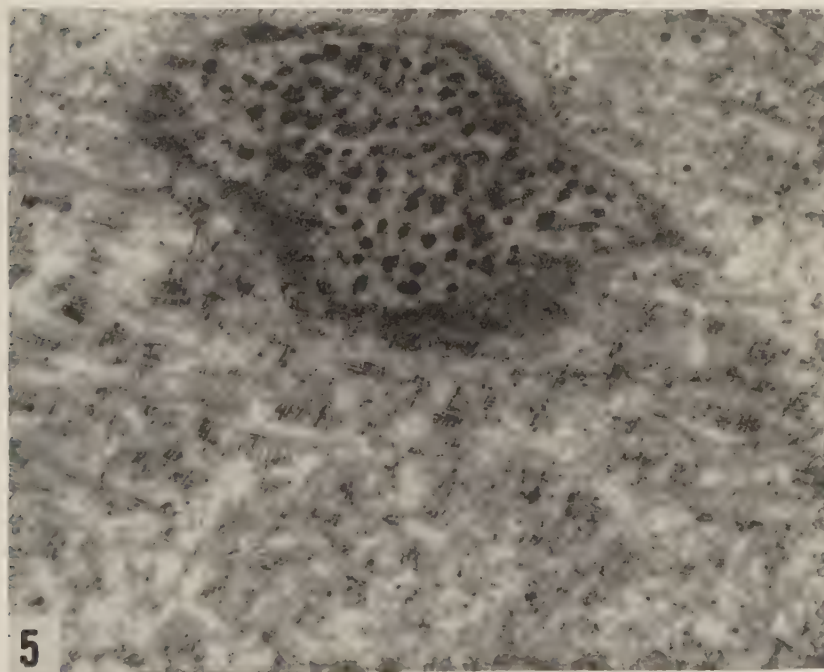
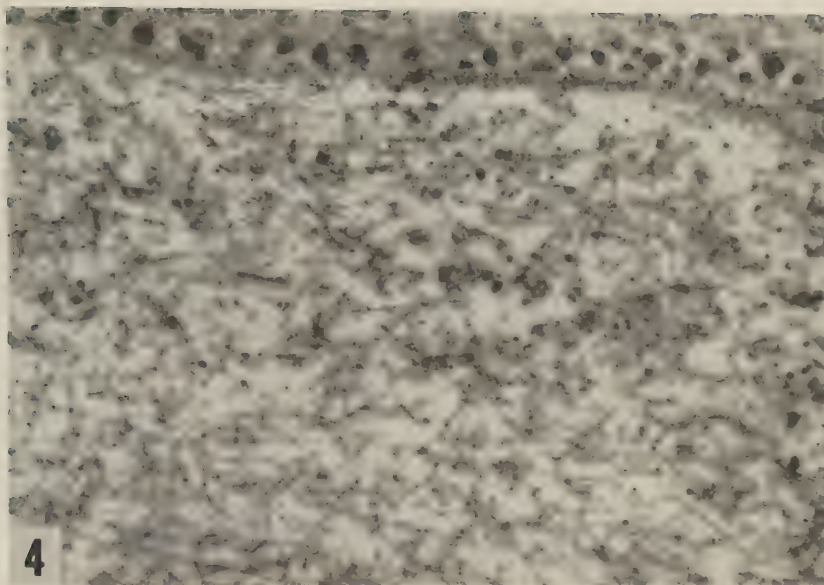


Fig. 4 Basement lamella in process of reconstruction, 11 days after wounding. $\times 13,500$.

Fig. 5 Tangential section near epidermal surface of same preparation as that shown in figure 4. $\times 17,000$.

Now, if at this stage a tangential section is laid through the slightly curved underside of the epidermis, the reorientation of the fibers in this zone becomes quite conspicuous (fig. 5). Surrounding the sliced-off cap of epidermal cell, with its dotted appearance, we see here about five consecutive layers in which the fibers have already assumed parallel alignment with the characteristic alternation of fiber directions by approximately 90° from layer to layer.

It is noteworthy that these fibers, while still only about 200 Å in diameter, are spaced about 500 Å apart, and that despite this lateral separation, they are already in lateral register. Considering that the actual periodicity is of about the same order of magnitude as the lateral separation, one gets the impression that each successive layer of fibers is being superimposed upon the preceding one in the manner of cross-ties being laid along lines connecting the nodal points of the fibers of the preceding layers.

These are the facts. As for an explanation, one might assume that the first ordering step consists of the alignment of fibers nearest the epidermal cells in one plane, with their cross-bands being forced into register. This process would then repeat itself in the next layer, which would become stacked up on the former by the nodal points of its fibers coming into equilibrium positions when reaching alignment with the nodal points of the fibers of the already settled layer. Evidently, this would give them two degrees of freedom, either parallel or at right angles to the former layers, and if we assume that for some reasons of symmetry and equilibrium only the orthogonal position would be permissible, we would have at least a formal interpretation of the basic pattern of the membrane, the geometric element of which would be a cube of 500-Å side length.

The process of ordering definitely proceeds into the membrane from the epidermal side downward, and layer after layer assumes definition by the ordering of preexisting, previously disoriented fibers. This process is accompanied by

the growth of the fibers into the 500-A class and by an increase in the density of the packing, apparently through the accretion of other fibers. Just how these further steps are achieved is still unknown.

The crux of the phenomenon, however, is that whereas the fibers are derived from the underlying mesenchyme cells as submicroscopic entities, and are present already in the polymerized form but in disoriented patterns, geometrical regularity arises from some ordering contact interaction with the overlying epidermal cells and sweeps the less-ordered fiber mat into a fabric of higher-order regularity. It is essentially this principle of the emergence of a higher-order regularity from preformed macromolecular complexes, rather than directly from molecular solution, that I wanted to emphasize with this example. It is the type of principle for which we have as yet no proper explanation in terms of the lower-order events. The problem is further obscured by the possibility that the macrocrystallinity to which we have alluded is perhaps not even a property of the electron-microscopically conspicuous fiber system, but on the contrary, of the electronoptically unresolvable ground substance in which the former lies embedded, with the fibers merely rendering visible a lattice pattern of their surroundings to which they have conformed.

Although the story I have presented here has for the first time traced the origin of such an orderly fabric, the sheer existence of similar fabrics has been established before (electronmicroscopically) in a few other instances; e. g., the cuticle of the earthworm (Reed and Rudall, '48), the peritrophic membrane in the intestine of insects (Mercer and Day, '52), and some of Frey-Wyssling's pictures (this symposium) of the plant cell wall certainly offer related aspects of the common problem of how tissue texture is woven from its constituent macromolecular threads. This promises to become a fertile field of borderland investigation.

That the principle of *orthogonal* arrangement of adjacent layers of fibers is of rather general occurrence, is indicated by some observations made on abnormal muscle fibers (Weiss and James, '55), in which a central longitudinal core bundle was found to be surrounded by peripheral coils of fibers, wound circumferentially around the former. The axes of the two sets thus included an angle of 90° . The fact that in these aberrant cases likewise, the homologous segments of all fibers of either system were in register, resulting in the appearance of over-all cross-striation, emphasizes the strength of the forces that produce the lateral alignment of molecular chains with periodic axial repeat patterns.

The purpose of my discourse has been to exemplify, in a clear-cut case, the type of problems that we shall have to face if we are to bridge the gap between the molecular and organismic dimensions by a continuum of knowledge rather than a string of words. We still must turn to the organism for furnishing us with the proper objects of study and biologically meaningful objectives, but if we search diligently, we shall find more objects matching the relative simplicity of the one here described; we shall thus escape the dilemma of having to choose between the forbidding inscrutability of "the organism as a whole" and artificial and absurd over-simplifications.

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ASPECTS OF MACROMOLECULAR ORIENTATION IN COLLAGENOUS TISSUES

J. T. RANDALL

*Wheatstone Laboratory and Medical Research Council,
Biophysics Research Unit, King's College, London*

SEVEN FIGURES

INTRODUCTION

This paper will review three problems investigated in the Wheatstone Laboratory that have a direct relation to the discussion of macromolecular fabrics and patterns. The first investigation is of fibrogenesis *in vivo*; it deals with the site of formation of collagen molecules and the subsequent formation and growth of tendon fibrils and fiber bundles (Fitton Jackson, '55, '56). The second study is of the fine structure of developing bone; a predominating feature of this work is the associated accumulation of crystalline particles of hydroxyapatite and the morphological relation of these particles to the fine structure of collagen fibrils (Fitton Jackson, '57a; Fitton Jackson and Randall, '56). The third problem concerns the fine structure (Fitton Jackson, '57b) and biochemistry (Watson and Smith, '56) of the cuticle of annelids, particularly of *Lumbricus*; though the earth worm cuticle does not possess many of the characteristics of vertebrate collagen, it nevertheless is included within this class of proteins (Astbury, '47).

FIBROGENESIS *IN VIVO*

The fine structure of developing tendons of the metatarsal rudiment of avian embryos and young fowls has been investigated by the use of thin sections as an example of tissue in which the collagen bundles are well orientated.

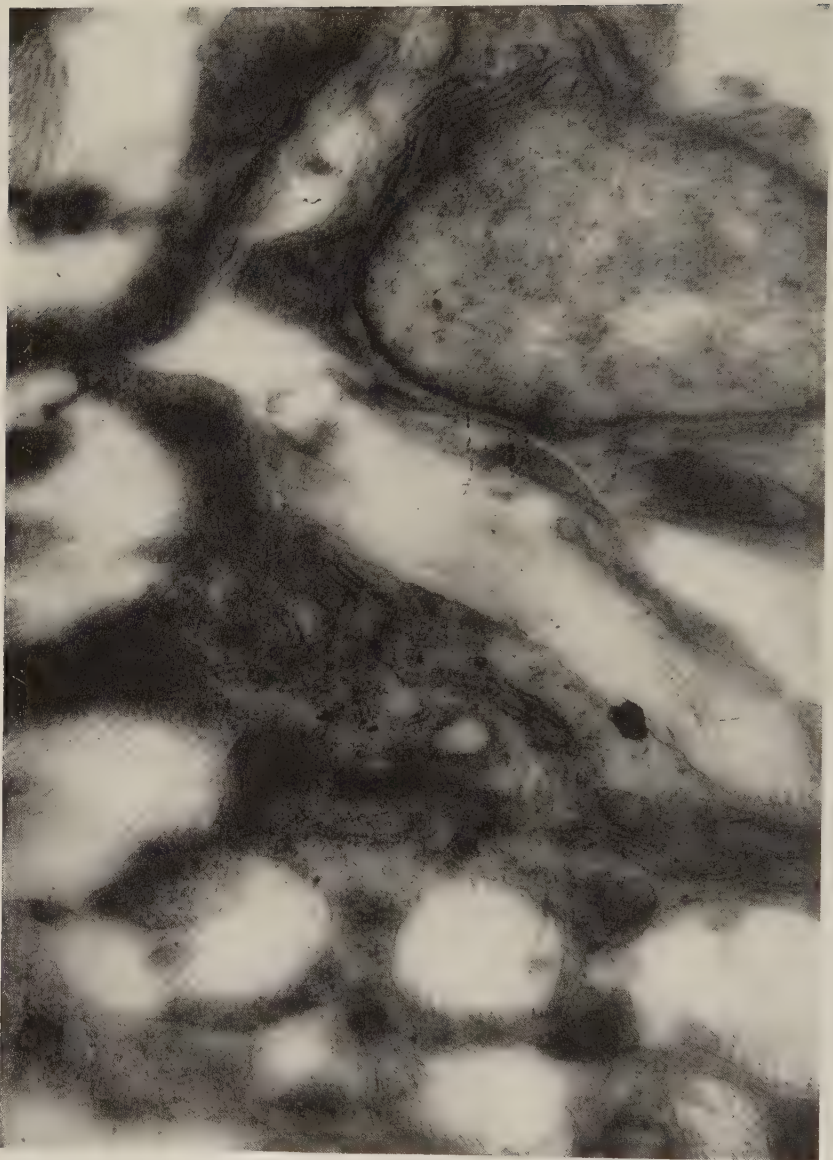


Fig. 1 Electron micrograph of a section of 10-day embryonic tendon showing that the intercellular areas are fairly well defined and contain many fine fibrils that are in close association with the cells. ($\times 30,000$)

Previous work (e.g., Porter, '52; Wassermann, '51, '54; Wyckoff, '52) has supported the suggestion that collagen fibrils originate in association with the cell surface. It was further generally assumed that the fibrils, once formed, enlarged outside the cells though the mechanism whereby this occurred was not elucidated.

The studies on the morphogenesis of tendon recorded in the paper by Fitton Jackson ('56) have shown that newly differentiated fibroblasts first form into protoplasmic sheets; in an 8-day embryo, filaments 80 A in diameter are located throughout the tissue except in the nuclear areas; the filaments are usually in small groups of up to twenty. After 9-10 days' incubation, parts of the cell surfaces are distinguishable and intercellular regions develop (fig. 1). The number of filaments has increased considerably and they are found to be arranged in two ways; in transverse sections small groups of filaments (represented by dots of about 100 A diameter) lie in areas that are undoubtedly cytoplasmic, but more numerous and larger groups of filaments are observed in the intercellular regions. These latter groups are normally bounded on at least one side by the cytoplasm of the adjacent cells, whereas the rest of the group merges into the structureless substance of the extracellular area that presumably contains intercellular fluid.

At 11 days, a periodicity characteristic of immature collagen may be distinguished in the filaments of longitudinal sections of the tendon. The filaments may therefore be identified as collagen fibrils, an interpretation supported by high-angle X-ray diffraction in which the tissue at this age gives a recognizable fiber diagram for collagen.

At 14 days' incubation, the intercellular regions have increased considerably and are packed with fibrils of 250 A diameter (fig. 2). If the surfaces of the cells are traced, it may be seen that the collagen fibrils have formed into bundles that are interlocked between the cells; the extracellular areas, however, are connected with each other. Each fibril is clearly

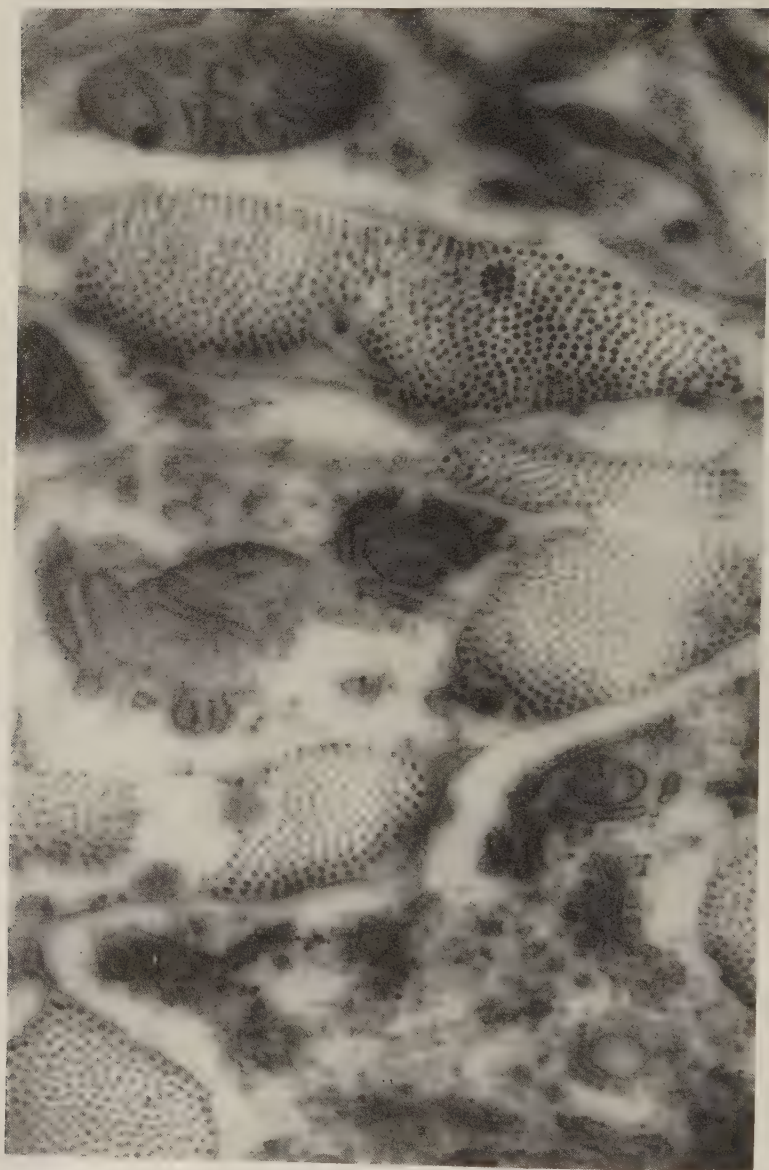


Fig. 2 Transverse section of tendon at 14 days incubation. Intercellular spaces have increased and become filled with collagen fibrils, each of which is invested by interfibrillar material. Some of the cytoplasm of three cells is shown, and at the top, left, of the micrograph, a mitochondrion is visible. ($\times 30,000$)

surrounded by a less dense substance, the interfibrillar material. In transverse sections the area of this material in proportion to that of the fibrils is less than the corresponding ratio for 11-day tendon.

The amount of extracellular material continues to increase with advancing age. At 20 days, the normal periodicity of 640 Å of the fibrils is apparent, and the average diameter is 400 Å; the relative amount of interfibrillar material has

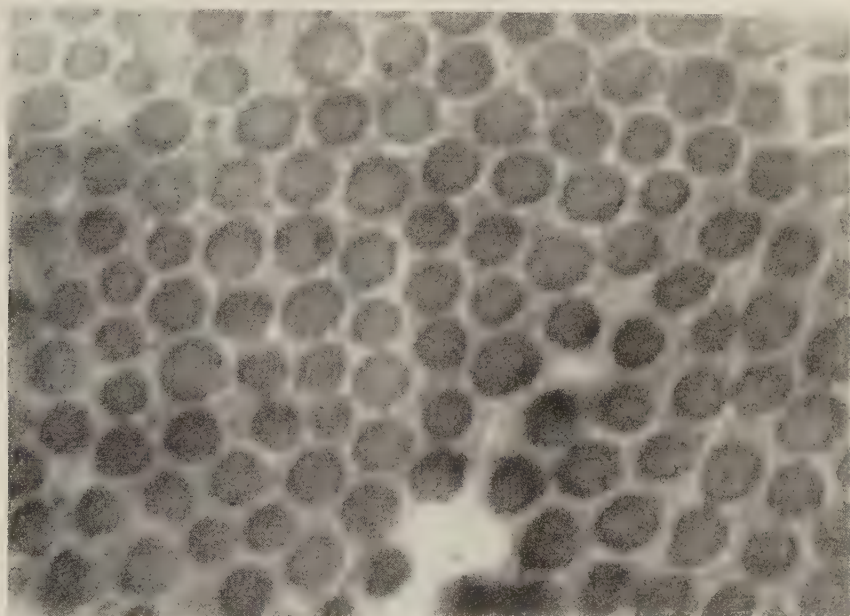


Fig. 3 Transverse section of tendon from an adult fowl. Each fibril has a distinct outer boundary. ($\times 100,000$)

again decreased. Fine cytoplasmic processes from adjacent cells extend outward between the bundles and surround them, the processes mingling with each other. Bundles of collagen fibrils of young fowls consist of a condensed mass of fibrils with a mean diameter of 750 Å (fig. 3), but each fibril is still surrounded by interfibrillar material; within each fibril smaller denser units are visible, which may possibly represent subfibrils.

Thus it is evident that the diameter of the fibrils steadily increases with age (fig. 4); the most rapid increase occurs between the twelfth and eighteenth days of embryonic life. It has also been shown that as the diameter of the fibril increases, the relative cross-sectional area of the interfibrillar material within a bundle diminishes; the degree of close-packing of the fibrils within the bundles can be calculated from these measurements, and plotted as a function of age.

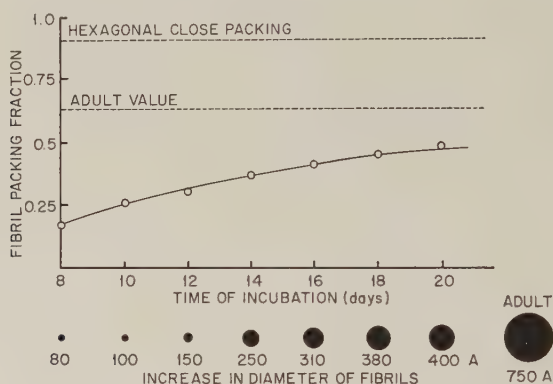


Fig. 4 Diagram of (1) the increase in the diameter of the collagen fibrils with age and (2) the curve of the packing fraction of the fibrils with age. The packing fraction of long solid cylinders in hexagonal array is marked for comparison purposes as well as the figure for adult tendon (0.62).

During the development of the fiber bundles, only small variations in the diameter of the individual fibrils are noticeable, which suggests that the fibrils must have been formed almost simultaneously or by a very strictly controlled mechanism. From the data obtained from the quantitative analysis of the developing tendon and from the corresponding X-ray fiber diagrams, Fitton Jackson has concluded that the interfibrillar material must contain collagen molecules or their precursors, which are subsequently laid down in an ordered way on the individual fibrils during the process of growth.

Histochemical observations on tendon tissue (Fitton Jackson, '55, '56) have shown that periodic acid-Schiff positive

granules are present in the cytoplasm of the fibroblasts from the eighth day of incubation onward. The granules also give a positive reaction with the Hale technique for demonstrating acid-polysaccharide and with the Mazia technique for protein. Further cytochemical tests on freshly teased preparations of tendon have shown that the granules have metachromatic properties in the living state, and a *pH* of about 7. Granules have not been observed in either the living or fixed cells from tendon of adult fowls. The fibroblasts are strongly basophilic in most of the cytoplasm, which indicates the presence of ribonucleoprotein, and the cells also show alkaline phosphatase activity at about the ninth day, the intensity of the stain increasing with the deposition of the fibrous material.

Fitton Jackson has postulated that the fibrogenic cell plays a double role in the synthesis of collagen and that the cytoplasmic granules together with other cytoplasmic organelles produce both polysaccharide and protein; the way in which the intercellular material differentiates suggests that it is formed when these cellular products are secreted and come into contact with the interstitial fluid. The author has pointed out, however, that the morphological picture indicates the collagen fibrils are formed both intra- and extracellularly, and suggested that this transformation could occur by the cellular secretions being extruded from the cell into the intercellular region and there reacting with the fluid, or the cell secretions might come into contact with fluid contained within the cytoplasm owing to pinocytotic activity (Lewis, '41), which might account for the apparent intracellular formation of fibrils.

THE FINE STRUCTURE OF AVIAN BONE

Electron microscope and histochemical observations have been made by Fitton Jackson on the differentiation of the shaft of the metatarsal rudiment, the frontal bone rudiments, and the angulare and supra-angulare bone rudiments of the mandible of embryonic fowls from the fourth to the twentieth day of incubation. The metatarsal rudiment was chosen for

the study of periosteal and endochondrial ossification and the other tissues for the investigation of ossification of membranes.

The morphological features of osteoblasts are essentially similar to those of fibroblasts (Fitton Jackson and Randall, '56; Fitton Jackson, '57a). The cytoplasm contains paired lamellae with associated particles adjacent to the exterior surface of each lamella; the substance lying between each lamellar pair is denser than the surrounding cytoplasmic matrix. In places, the cytoplasm often contains small particles of less than 40 A that are formed into particulate structures rather similar in outline to the image of the lamellar pairs. Many mitochondria are in evidence and their structure is similar to the structures of other metazoan cells. Each osteoblast contains a zone in which small vesicles, composed of an outer membrane confining a substance of lower density, are associated with larger bodies or vacuoles. Each of these larger bodies is delineated by a double membrane seen as two lines about 40 A wide and separated from each other by about 100 A. This zone of the cytoplasm may represent the Golgi complex (cf. Sjöstrand, '55). The histochemical properties of osteoblasts are similar to those of fibroblasts, as described in the first part of the paper.

The elaboration of collagen fibrils in bone-forming tissue is essentially similar to that in the tendon, but the diameter of the collagen fibrils adjacent to the cells in periosteal bone is about 400 A and there is an axial periodicity of about 640 A with varying degrees of fine structure. Transverse sections confirm that each fibril is invested by less dense interfibrillar material. Each osteoblast becomes surrounded by bone matrix, owing to the continual deposition of organic material; thus the cell is transformed into an osteocyte. Cytoplasmic processes run from the surface of the young osteocytes into the extracellular material to form canaliculae that may be connected with those of neighboring cells.

The beginning of the calcification of the organic matrix is marked by two processes; small particles of less than 100 A

become arranged in an organized fashion in contact with the collagen fibrils (fig. 5), and most of the fibrous matrix becomes embedded in an opaque material. On analysis (Fitton Jackson, '57a), it is found that the particles are localized in a ring around the collagen fibrils almost exclusively between the d and ab bands (notation of Schmitt and Gross, '48, and Grassmann, '53) of the main period. Since it is evident that in some of the micrographs the fibrils are seen in longitudinal

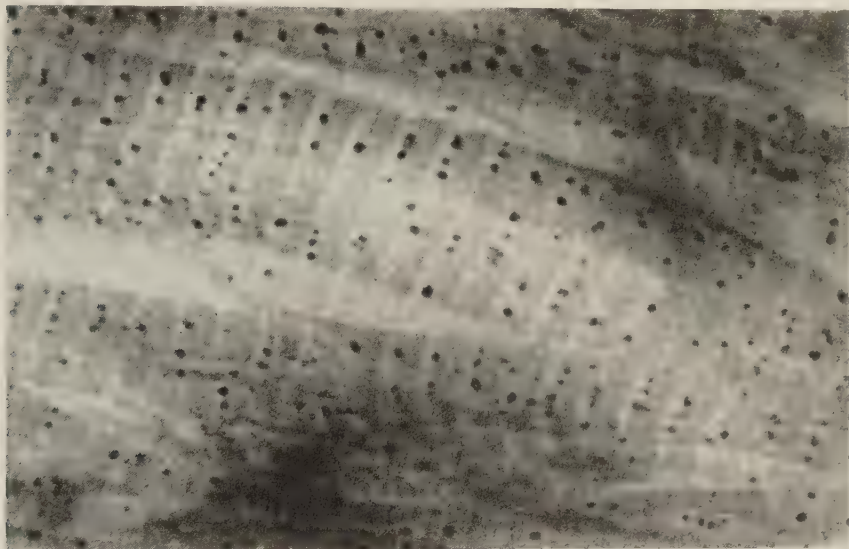


Fig. 5 A section of periosteal bone from a 16-day embryo, showing the periodic structure of the collagen fibrils; particles of less than 100 Å in size are localized in one interband of each period. ($\times 110,000$)

section, it follows that the particles may not only be on the surface of the fibrils but may be embedded within the fibrils in an organized fashion. So far as can be observed there is no preferred orientation of the particles relative to the fibrillar axis. By the use of the technique of electron diffraction, powder diffraction patterns have been obtained from sections in which the particles are visible and measurements of the Bragg spacings of these diffraction rings have identified the

material as an apatite. The particles must be related, therefore, to the deposition of the apatite content of the bone (Fitton Jackson, '57a).

Since the localization of the particles within the fibrils between the d and ab bands, i.e., within the major region of indentation of the period, is so precise, Fitton Jackson suggested that the site of the particles may be attributable solely to mechanical factors, or specific chemical groups in the inter-band region may be involved. The particles in embryonic avian bone are less than 100 Å in diameter, whereas in adult avian ossified tissue they are 220 Å by 73 Å (Finean and Engström, '53); it is obvious therefore that they must enlarge as ossification progresses. Thus the observations reviewed have shown that the formation of the organic matrix of bone is essentially similar to fibrogenesis in tendon, except that fibrils with immature banding have not been observed in the former. Furthermore, evidence has been provided to show that the collagen fibrils play a significant role in the crystallization of the inorganic component of bone matrix.

THE EARTHWORM CUTICLE

Collagen protein is widely distributed throughout the animal kingdom (Marks *et al.*, '49). High-angle X-ray diffraction has shown that the earthworm cuticle contains collagen (Astbury, '47), but the characteristic axial periodicity (about 640 Å) of the constituent fibrils is apparently lacking (Reed and Rudall, '48). Proteins identified as collagen on these criteria are often termed "secreted collagens" (Bear, '52).

*Fine structure of the cuticle (Lumbricus sp.).*¹ The cuticle of the earthworm covers a single layer of columnar epithelial cells; these cells have a granular cytoplasm and are somewhat similar in appearance to the goblet cells of the mammalian intestine. The fine structure of the cytoplasm seen in the electron microscope shows that it is highly vesiculated. Some bodies, which may be identified as mitochondria, contain many

¹ Work carried out by Dr. S. Fitton Jackson.

parallel membranes and are similar in structure to the Type I mitochondria of protozoa described by Randall ('57). In transverse sections, the periphery of the cells adjacent to the cuticle is composed of many evenly spaced folds that appear to taper into fine cytoplasmic processes that penetrate the cuticle and are connected with the exterior membrane of the worm.

Thin sections of the cuticle show that it is composed of five regions; the lowest layer, adjacent to the main body of the

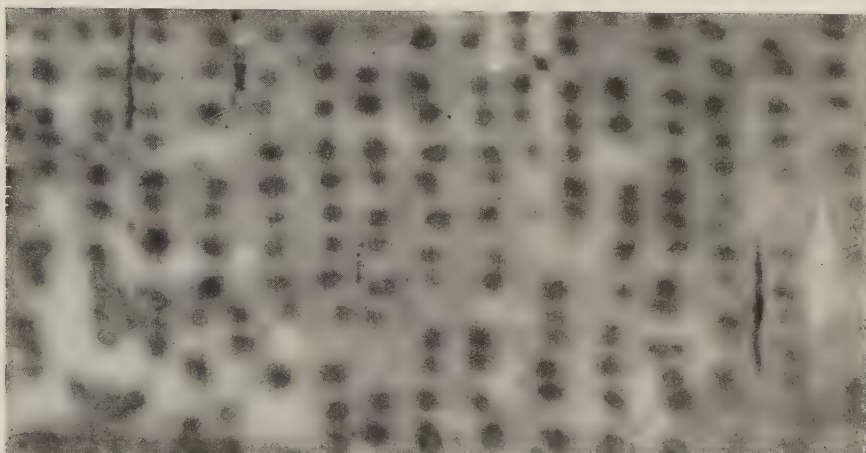


Fig. 6 Oblique section through the lowest layer of the earthworm cuticle. ($\times 30,000$)

epidermal cells, contains the base of the cytoplasmic processes described. In oblique sections, dense oval structures (which may represent these processes cut in transverse section) are arranged in rows and are usually about 700–1000 Å in diameter and 2000 Å apart (fig. 6). Each row appears to be contained within a ribbon of less-dense material about 2500 Å wide. These structures may have appeared as the granular layer recorded by Reed and Rudall ('48) in their work on replicas of earthworm cuticle.

The region immediately above this deepest layer contains fibrils about 1000 Å in diameter, and in suitably orientated

sections the cytoplasmic processes are visible; there are about four layers of fibers in this region. The middle region of the cuticle is usually about 2μ thick; it is composed of about eighteen layers of apparently unbanded fibrils about 1500–2000 Å wide and orientated in a crisscross fashion at angles 74 – 106° to each other. The fibrils are embedded in an amorphous material, however, that tends to make precise obser-

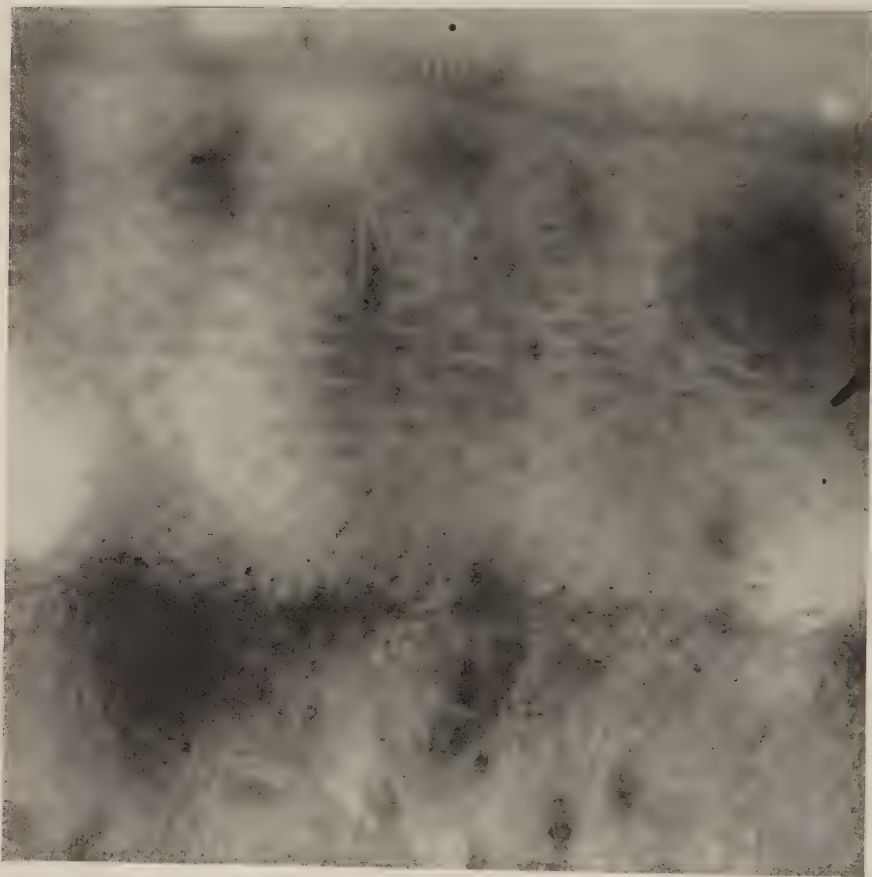


Fig. 7 Near-transverse section of the cuticle, which shows the layers of fibrils that lie adjacent to the epidermal cells. The microvilli on the outer surface of the worm and the cytoplasmic processes that stretch between the cells and the outer membrane are seen. ($\times 30,000$)

vation difficult. In transverse sections (fig. 7), the fibrils of the cuticle appear to weave in and out of the perpendicularly arranged cytoplasmic processes that stretch between the cells and the outer membrane. Thick sections show that the pattern is like that of a basket weave.

Just beneath the outer layer, a few fibrils can sometimes be distinguished, but more often only a thin amorphous stratum is seen. The outer layer of the cuticle is composed of a double membrane, with numerous microvilli about 1500 Å long and 500 Å in diameter (fig. 7). Reed and Rudall ('48) defined these microvilli as a corpuscular layer, but observations on transverse sections of the cuticle show that the exterior of the worm is undoubtedly covered by a system of microvilli. This finding may account for the presence of the numerous fine cytoplasmic processes that penetrate the cuticle, for it is unlikely that the microvilli could exist in an extracellular position.

The observations that have so far been made suggest that the precisely arranged cytoplasmic processes may take part in the orientation of the unbanded fibrils, for it is reasonable to assume that the epidermal cells are responsible for the secretion of the precursors of the cuticle.

*Amino acid analysis.*² In view of the lack of axial periodicity in the fibrils of the cuticle, it is interesting to try to determine what are the common chemical features that underlie the constancy of the three-dimensional molecular structure implied by the similar high-angle X-ray diffraction patterns obtained both from the cuticle and from collagens derived from vertebrate sources (e.g., Bear, '52). Since the cuticle is easily obtained in amounts that permit chemical analysis by modern micromethods, the chemical composition of this material has been investigated (Watson and Smith, '56).

Cuticles are gathered for analysis by stripping them from earthworms (*Lumbricus* sp.) drowned in ether. Analytical

² Work carried out by Mr. M. R. Watson and Dr. R. H. Smith.

results for several preparations made in this way agree within the limits of experimental error for the particular determination. The cuticle consists of a protein, which accounts for some 80% of its weight, and a non-nitrogenous polysaccharide. The protein closely resembles the mammalian collagens in the proportions of those amino acid residues that have nonpolar side chains, in the high glycine content, and also in having a high content of hydroxyproline, in imino acid that does not occur to any appreciable extent in any class of protein except the collagens. The hydroxyproline content of the cuticle is extraordinarily high, some 50% higher than the amount normally present in a mammalian collagen. The hydroxyamino acids, serine and threonine, are also present in much greater proportions in the cuticle. The composition thus rather resembles that of a typical mammalian collagen that has been hydroxylated throughout in a most general manner. In this connection it may be significant that the proline content of the cuticle is very low, suggesting that the hydroxyproline has been formed at the expense of proline.

The other major difference between the composition of earthworm cuticle and that of the mammalian collagens is the paucity of basic amino acid residues in the former, arginine especially being present in very small quantities. The balance of charge in the protein appears to be maintained by the large proportion of carboxyl side chains present in the amide form.

The interpretation of the relation of these chemical analyses to the observed X-ray diffraction and electron microscopy of the cuticle is as yet obscure. It is hoped, however, that further work on collagens from invertebrate sources will help to elucidate this problem.

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ORGANIZATION IN BIOCOLLOIDAL SYSTEMS

GERALD OSTER

Polytechnic Institute of Brooklyn, Brooklyn, New York

ONE FIGURE

INTRODUCTION

There are numerous examples of organized structures in living systems ranging from the 10-m μ region as in the periodic structures in collagen (for review, see Bear, '52) to the micron region as in crystalline arrays of bacteria (Goldacre, '54). The most elaborate organization is that revealed by the precise movement of chromosomes during mitosis and meiosis.

In this paper I shall attempt to explain in physicochemical terms how some of these structures might arise.

LONG-RANGE PERIODICITY IN FIBERS

X-ray and electron microscope studies have revealed that periodic spacings of about 10 m μ exist in many natural and synthetic fibers (for review, see Hess, '54). Even in synthetic fibers, where the monomeric units are all of one kind, such long-range periodicities do occur. High polymers of the random chain form will become entangled when the molecular weight exceeds a critical concentration (in weight percentage) given by $1.6/[\eta]$ where $[\eta]$ is the intrinsic viscosity of the polymer (Nishijima and Oster, '56a). Such entanglement is revealed by an increase in local viscosity as the concentration of the polymer is increased. Entanglement occurs in the outer fringes of the random coils so that if a solution of the polymer at a concentration exceeding the critical value is now spun to make a fiber, the resultant fiber will have regions of crystallinity where the entangled portions have become aligned.

Such regions will be separated by amorphous coiled polymer chains. Calculations (Nishijima and Oster, '56b) show that periodic spacings should occur at the distance observed by X-ray diffraction and the changes in spacing with treatment of the fiber are in agreement with the observed results. It is not known whether natural fibers are formed by a spinning process (with silk this is the case, of course), but if so, our theory would explain how large spacings arise from natural polymers containing small monomeric units.

ORGANIZATION FROM IONIC ATMOSPHERE REPULSION

Charged colloidal particles in solution are surrounded by a diffuse atmosphere of predominantly opposite charged ions. The average thickness of this double layer (in millimicrons), is, according to the Debye-Hückel theory of electrolytes, given by $1/\kappa = 0.3/\sqrt{C}$ where C is the molar concentration of the salt (here taken to be a 1 — 1 electrolyte). As a consequence of this double layer the colloidal particles will repel one another. Detailed theory (Verwey and Overbeek, '48) shows that the repulsion force between charged parallel plates is proportional to the square of the potential on the plates times $e^{-\kappa R}$ where R is the distance between the plates. For parallel rods calculations show that the repulsive force falls off as $\coth(\kappa R)$. The repulsive force has the effect of increasing the effective size of the particles as determined by the deviations of the properties of the solution at finite concentration from thermodynamic ideality.

At very low salt concentrations the system may exhibit structural rigidity, which is destroyed on decreasing the thickness of the double layer by increasing the salt concentration (Oster, '50). Protein solutions with very little salt present exhibit an ordering that can be destroyed when more salt is added (Doty and Steiner, '49). Lowering the salt concentration increases the apparent volume of the particles and the system now has more order. A crude analogy would be the following: If in a crowded room with doors locked, the

people were asked to keep as far away from their nearest neighbors as possible, they would then form a military (crystalline) array. It is not necessary to postulate the existence of long-range attractive forces in order to account for an ordered colloidal system.

The long distance, two-dimensional order exhibited by concentrated solutions of tobacco mosaic virus (Bernal and Fankuchen, '41) can be explained in terms of the repulsive forces discussed above. A simple geometrical argument shows that if the rod-shaped particles fill the space uniformly in two dimensions, the distance R between the centers of the particles for a solution of weight percentage concentration C is given by $R = 9.4 \sigma / \sqrt{\bar{V}C}$ where σ is the diameter of the particles and \bar{V} is the partial specific volume. Thus for tobacco mosaic virus $\sigma = 15 \text{ m}\mu$ and $\bar{V} = 0.73$ so $R = 165 / \sqrt{C} \text{ m}\mu$, in exact agreement with X-ray diffraction data.

ORDERING OF ROD-SHAPED PARTICLES

Elongated particles will tend to be aligned parallel to one another in solution when the volume available per particle is nearly as small as the covolume of the particles. If the particles are highly elongated, such alignment will set in at relatively low concentrations since for stiff rods the covolume equals the axial ratio of the particles (for discs it is about two-thirds the axial ratio). In fact, theory shows (Onsager, '49) that when the concentration of the rods is such that the volume available per particle is less than about one-third that of the covolume, the system will separate into two phases — an isotropic phase where the rods are randomly oriented and an anisotropic phase where rods are arranged in a parallel fashion. Theory further predicts that the bottom phase will be 34% more concentrated than the top phase. These calculations agree with the experimental results (Oster, '50) if the covolume is taken to be that obtained from the covolume observed from light-scattering experiments. Thus it was found that the lower the salt concentration the greater is the co-

volume and, hence, the lower is the concentration for two-phase formation. Still further, it was found that for a polydispersed system of rods, the longer particles appear in the anisotropic phase as would be predicted from theory (Oster, '50).

If care is taken to obtain a monodispersed solution of tobacco mosaic virus rods, then another phase will separate from the anisotropic phase. This phase is also anisotropic and exhibits an iridescence caused by light diffraction between planes made by the ends of the rods (about 300 m μ in length). That is, the particles form a three-dimensional crystal (Oster, '50). This crystalline material, along with other crystal modifications, has been observed in the inclusion bodies of plant cells suffering from the virus disease (Wilkins *et al.*, '50). The existence of such crystals, which do not fill out the whole volume of the solution, demands that attractive forces must also be operative.

Small-angle X-ray diffraction studies of deoxyribonucleic acid (DNA) show that there is a high degree of ordering in solution (Riley and Oster, '51). All samples of varying degree of heterogeneity in molecular weight exhibit a diffraction spacing that is dependent on concentration according to the formula given for space-filling rods where σ , the diameter of the particles, is taken to be 1.6 m μ . Sedimentation data (Kahler, '48) give a diameter of 1.8 m μ if it is assumed that the particles are stiff rods and obey classical hydrodynamics. Unlike the case for tobacco mosaic virus, however, there is still another X-ray diffraction spacing for DNA. This spacing is also inversely proportional to the square root of the concentration but is two or three times as long, depending on the sample, as that of the shorter spacing. For a sample of very high molecular weight (Signer sample, molecular weight 3.3×10^6), the diameter σ is about 6 m μ and apparently represents a bundle of seven of the elementary rods. H. L. Nixon and I have actually observed such bundles in the electron microscope. More-degraded samples of DNA exhibit a spacing of the elementary rods equivalent to $\sigma = 1.6$ m μ as before,

but now the longer spacing is equivalent to $\sigma = 3.6 \mu$. Although the analysis of the X-ray data is not complete, we are certain that in DNA solutions there are two regions of inhomogeneity corresponding to the two spacings and these change with concentration of DNA in the same manner. It follows that there must be some attractive forces acting between the particles.

LONG-RANGE VAN DER WAALS FORCES

Van der Waals forces between two atoms have been shown by London ('37) to arise from the induced polarizability of one atom reacting on the other atom. The energy of attraction between atoms is short range and falls off as the inverse-sixth power of the distance, the proportionality constant being determined by the polarizabilities and the ionization potentials of the atoms in question. London-van der Waals forces are additive. Hence, the larger the particles, for a given distance of separation, the larger is the force of attraction. Furthermore, the forces between aggregates of attracting centers are of longer range (i.e., fall off with a smaller power of the inverse distance) than the separated attractive centers. Integration over spherical particles (Hamaker, '37) shows that the potential of interaction for spheres very close to one another is inversely proportional to $s - 2$ where s is the ratio of the center-to-center distance to the radius. When $s = 3$, the energy is inversely proportional $(s - 2)^2$ and increases in inverse power of $s - 2$ as s increases further and finally approaches the inverse sixth power for large separations. My calculations show that for parallel rods the energy of attraction falls off as the inverse fourth power of the distance. The proportionality constant for the energy of interaction is, for proteins or nucleic acid in water, about 10^{-12} ergs. Spheres separated by a center-to-center distance three times that of the length of the radius would attract each other with an energy about 100 times that of the disruptive thermal energy (note: at room temperature $kT = 4 \times 10^{-14}$ ergs).

Thus we would expect that London-van der Waals attractive forces would become operative when particles approach to within distances comparable with the size of the particles. Opposing the attractive forces will be the repulsive forces caused by the overlapping ionic atmospheres of the particles. Hence, aggregates of particles, be they virus particles or even bacteria, could form in solution. The stability of such aggregates would be determined by the difference in energy between the attractive forces and the repulsive forces as compared with the thermal energy.

COLLOID PHYSICS OF CHROMOSOME PAIRING

From a chemical point of view, the nucleus in living cells differs from the other portions of the cell in that it contains DNA and a very basic protein. In salmon sperm these substances are found in high concentration — 60% DNA and 20% protamine, a highly basic protein. In mammalian cells there may be about 15% DNA in the nucleus, but threads isolated from such cells contain about 40% DNA (Mirsky and Ris, '47).

The DNA concentration per cell, for a given species of animal, agrees with the number of chromosomes in the cell (Boivin *et al.*, '48; Mirsky and Ris, '51). Thus somatic (or body) cells contain about twice as much DNA as sex cells (gametes). Hence, DNA with its associated basic protein is reproduced every time the cell has divided (mitosis) and is reduced to half its normal value in the formation of sex cells (meiosis).

Watson and Crick ('53b) have proposed a model for DNA — a double-stranded helix — based on the X-ray diffraction data of DNA. They suggest that DNA serves as its own template and can reproduce in this manner (Watson and Crick, '53a). However, the problem is not only one of reproduction of DNA but also of its associated protein. Radioactive phosphorus is taken up by the nucleus just before division, and there is no uptake during division (for review, see Doniach *et al.*, '53).

That is, DNA is synthesized only during a short period just before division. Furthermore, radioactive sulfur is also taken up only during this period. Hence, the synthesis of protein accompanies the synthesis of DNA.

Since no DNA or protein is synthesized after the cell has prepared itself for division, we should expect that subsequent behavior of the DNA and the protein is governed by colloidal physical considerations. When the nucleus is in the resting stage, it is poorly stained by basic dyes (only one or more spots, the nucleoli, are usually visible). Presumably, the nucleic acid is in the highly swollen state that I have described. During division, however, pairs of highly stainable threads (chromatin) appear, which then thicken and eventually separate to form identical cell nuclei.

Details of the threads are observable in the early stages of meiosis (stages known as leptotene and zygotene). Here the chromatid threads appear on them as strings with beads (chromomeres) of different sizes. The actual appearance of the beads may depend somewhat on the chemical treatment (fixation) given the cell in order to enhance the staining.

Homologous chromatid threads (i.e., threads with the same arrangement of beads) line up together with the same beads of one member of the pair opposite the same beads in the other thread. As far as one can establish in the visible microscope, homologous chromomeres are not actually touching and the distances between the threads are slightly greater than the diameters of the larger beads, i.e., about $1\ \mu$. After examining many photographs of paired chromatid threads, I get the impression that ends where there are large beads are stuck together whereas ends where there are small beads are frayed. Cytologists who have observed pairing report that the pairing is a zippering action and that the zippering starts where the threads happen to be together in the nucleus (as in the bouquet effect) or where there is a high density of stainable material (i.e., where there are many large beads). After pairing, the

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threads split longitudinally (pachytene), and the threads of each new pair repel one another.

The high affinity of chromosomes for basic dyes shows that they bear a net negative charge (owing to the phosphoric groups of DNA) enabling their repulsion to be accounted for in terms of their overlapping counter ionic atmospheres. This repulsive force is a rather generalized one. But how we are to explain the nature of the long-range specific attractive forces that bring homologous chromosomes together is a question.

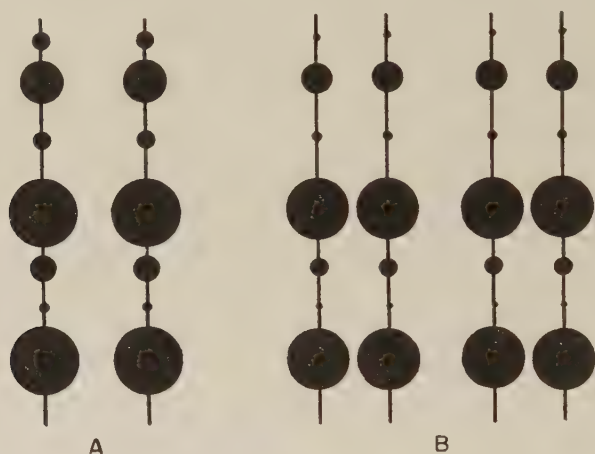


Fig. 1 Schematic arrangement of chromatid threads during meiosis. A, zygotene; B, pachytene.

As I indicated earlier, the long-range nature of additive van der Waals forces is highly sensitive to the geometry of the system in question; we would therefore expect that such forces that must influence the pairing would depend on the number and size of homologous beads. In figure 1, A represents a particularly favorable case; the small beads are dragged along with the large ones. If we split the chromosomes (condition B of the figure), the range of the forces becomes shorter and within a pair there will be attraction, but one pair will attract another pair only weakly since the van der Waals forces are reduced and the repulsion caused by the ionic double layer

becomes predominant. The split threads would then go off in pairs.

Some other cytological phenomena can also be explained by this theory (e.g., zippering, deletions). What is particularly important to realize, however, is that the general over-all behavior of the chromosomes in pairing can be explained in terms of ionic double-layer repulsion and of additive van der Waals attraction. No recourse need be made to "tuned gene oscillators" (Muller, '41) and other very special types of physical notions. The data that cytologists give us (i.e., the morphological observations) are precisely *the* data that are used in our physical calculations. Thus the affinity by the chromosomes for basic dyes (or for Feulgen stain giving the distribution of DNA) gives us the surface charge distribution, and the morphology of the chromosomes gives us the distribution of van der Waals attracting elements.

GENERAL DISCUSSION

URETZ¹: I am not a cytologist but I have some evidence that might prove grist for Doctor Oster's mill. In tissue cultures of newt, a living cell can sometimes be found just at the anaphase moment in which there is an isolated chromosome that is not attached to the metaphase plate. If this chromosome is suitably oriented so that the two daughter chromatids can be seen lying side by side, very often at the instant of the anaphase break, these two chromatids, which are very close together and showing a just visible split, will leap apart about 1 or 2 μ all along their length.

I wonder if, with some order of magnitude calculations based on your model, you could account for the forces that were holding the two daughter chromatids together until some sort of signal came along and released them at the instant of anaphase break.

OSTER: I cannot do this calculation and, because I have no data, I cannot do the reverse process of saying how long they

¹ R. B. Uretz, University of Chicago.

will hold together. But I can say once the chromatid thread is split, then I think I can calculate whether they go apart. That is the only part of the calculation I can make.

URETZ: Do you think your forces will hold over distances of $\frac{1}{2} \mu$ or so, which is the distance apart the two daughter chromatids appear to be?

OSTER: Yes, I have detailed calculations of these beads. They are very easy to make, by the way, because you soon find out that the farther along the chain they are the less they contribute to the attractive force. So you really have to account for only four or five beads and that takes care of the major portion.

In general, I should be able to account for the phenomena that you have described but I could not calculate what energy is needed to split them in the first place, unless you want to take the theory complete and go backward. But it is probably not held together, as an intact chromatin thread by these kinds of forces.

JEHLE²: Just two short remarks about Doctor Oster's lecture. I want to remind that it is the Dutch school to whom we owe the understanding of this interplay between van der Waal's forces and the forces that come from the electrostatic repulsion modified by the Debye-Hückel-Onsager layer, particularly to Hamaker, De Boer, Verwey, and Overbeek, and in the early days Kallmann and Willstätter, Derjaguin, and Vinograd.

Secondly, as to the application of this mechanism to the meiotic pairing, it was back in 1922 that H. J. Muller, the geneticist, pointed out the importance of this problem. Several decades ago he recognized that the motion of chromosomes during meiosis is determined by a highly specific attraction, which we think is of the London-van der Waal's type, and a repulsive force that can be regulated by ionic concentration changes of the medium.

² Herbert Jehle, University of Nebraska.

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INTERACTIONS OF ELONGATED PARTICLES

A DISCUSSION

TERU HAYASHI

Department of Zoology, Columbia University, New York City

Being a person trained in old-line biology I belong to that group that lives in some awe of such strange species as physical chemists, and I sometimes wonder how I managed to get myself into a position like this where I am standing in a denful of this strange species to act as a discussant. Nonetheless, at the risk of radicalism, I am going to depart from the usual role of a discussant and actually discuss the paper that was just given.

As nearly as I understand it, Dr. Oster's paper is based on the postulation of two sets of forces. The first is a set of repulsive forces caused by the existence of ionic double layers of like charges, and these are relatively strong forces. The second is a set of attractive cohesive forces — van der Waals forces. Although the latter forces in themselves are weak and short range, they are also cumulative; macromolecules or aggregates of molecules, they have the possibility of becoming long range and of considerable magnitude. These cumulative forces are complicated by the geometry of the particles involved, both by the shape of the particles and the distances between them.

Now, whatever may be said about the details of the hypotheses proposed by Dr. Oster, one important general feature stressed by the speaker that I, as a biologist, appreciate is that very precise and ordered structures can be obtained spontaneously from the interplay of physical and chemical

forces. This point was illustrated so spectacularly by Dr. Schmitt in this symposium with the collagen and paramyosin systems, and Dr. Oster illustrated it in his *in vitro* experiments. I think this point of view is becoming more and more fashionable — and it is quite satisfying to the biologist.

But with regard to the application of these forces to the very difficult subject of chromosome movements, it seems to me that Dr. Oster in his discussion was strictly limiting himself to what the cytologists refer to as the initial stages of meiosis — the leptotene stage, in which the chromosomes are rather thin, attenuated, and unpaired, becoming visible but already with certain organization, such as chromomeres, quite apparent. This is followed by the zygotene stage in which the point-to-point pairing takes place and then the pachytene stage in which each of the paired homologous chromosomes is split.

According to Dr. Oster, the zygotene pairing occurs because of the cumulative van der Waals forces, which are long range because of the size and shape of the chromomeres, and in the final paired state there is a critical balance between the attractive and repulsive forces, so that the paired strands do not actually touch. Subsequently the pachytene splitting reduces the attractive forces because the size of the particles involved is reduced, and the repulsive forces push the paired chromosomes apart.

Many kinds of evidence in support of this simple system of interacting attractive and repulsive forces, sometimes in surprising detail, may be found in the literature. For example, although Dr. Oster did not talk about mitosis, application of his scheme to mitosis results in a surprisingly good fit. Since mitosis differs from meiosis essentially in that the homologous chromosomes do not pair, we might ask why pairing does *not* occur, if we have such forces as attractive van der Waals forces. The answer seems deceptively simple. In mitosis, there is a precocious splitting of the individual chromosomes, so that when the chromosomes first appear in a stage cor-

responding to the leptotene stage, each of the homologs is already split. Being already split, according to Dr. Oster's scheme, the attractive forces are reduced because of the reduction in size, and although perhaps they may be sufficient to hold together the two halves of the split chromosome because of their proximity, they are not strong enough to attract a second split homologous chromosome. Thus pairing would not occur in mitosis.

Of course I am interpreting, but Dr. Oster's mechanism might plausibly explain such a fundamental difference. I think, however, that since the subject of cell division and chromosome movement is so complex and full of strange features depending on the particular species of cell observed, Dr. Oster would agree with me that his postulated mechanism is essentially only a first attempt to explain one fundamental aspect of meiosis — chromosomal pairing.

On this point, moreover, one might well ask whether such a simple mechanism is sufficient to account for the precision of the point-to-point pairing, a feature of meiosis of considerable biological importance. It is difficult to see how a generalized mechanism of attractive forces can account for the specificity of the pairing of chromosomes, unless further assumptions are made.

When we consider the electrostatic charges of the DNA molecules, we are tempted to speculate whether these charges are involved in a further aspect of chromosomal behavior, the shortening and elongation of the chromosomes at different stages of the division cycle. As presented by Dr. Doty and Dr. Schachman in this symposium, we have evidence that DNA behaves like a polyelectrolyte in solution, and that with a reduction in pH there is a shortening and coiling, or contraction, of the molecule and an elongation upon reversing these conditions.

Biologically, one might try to associate this evidence with such behavior of chromosomes, except, of course, that the chromosome is not DNA, but primarily DNA and protein organized

in some very precise way. Technically, formation of pellicular fibers of nucleohistone is a simple matter. Such fibers have a considerable tensile strength, indicating some sort of intermolecular bonding. If a fiber is then placed in a horizontal position between supports, with a considerable slack, and immersed in a medium of low pH , the fiber will contract and shorten, as indicated by the disappearance of the slack in the fiber. If the pH is raised, the slack appears again, showing the pH -induced contraction to be a reversible process.

This simple observation shows that DNA-protein behaves very similarly to DNA, and one is tempted to invoke the same forces. It may also be pointed out that this shortening is an entropic one; that is, entropic forces are involved. The subsequent elongation is an active elongation; that is, the fiber pushes itself out to the original length by the interaction of mutually repulsive forces.

The application of this mechanism to chromosomal changes is difficult, however, for the ionic changes involved seem to me to be nonphysiological. This illustrates the point stressed by Dr. Weiss (this symposium) that mechanisms based on findings from *in vitro* systems have their greatest difficulties when face to face with biological realities.

A MECHANISM FOR THE FORMATION OF FIBRILS FROM PROTEIN MOLECULES

DAVID F. WAUGH

Department of Biology, Massachusetts Institute of Technology, Cambridge

NINE FIGURES

There is considerable evidence that suggests, universally so far as one can tell, that fibrous proteins such as muscle, collagen, and keratin are constructed of small corpuscular units. These units can be observed under appropriate experimental conditions that are, in general, foreign to the environmental conditions provided by the organism. Consequently, under physiological conditions all the structures chosen as examples are too insoluble to allow detection of monomers in equilibrium or of precursor molecules. The mechanism of formation of fibrils therefore remains in question, and instructive information may be gained through studies of appropriate systems whose properties will allow deductions about mechanism. Thus knowledge of the mechanism responsible for the transformation of insulin into insulin fibrils may contribute to an understanding of the chemistry of insulin and of the possible mechanism of formation *in vivo* of stable and ephemeral fibrils.

The formation of insulin fibrils may conveniently be produced by heating solutions of 2.0% insulin at pH 1.5–2.0. The resulting fibril suspension has been found, through examinations of flow double refraction (Waugh, '44) or of electronmicrographs (Farrant and Mercer, '52), to consist of a remarkably uniform population whose members are approxi-

mately 150 Å in diameter and 40,000 Å long. The reactions leading to fibril formation with insulin are highly specific, as is indicated by their use as the basis for an *in vitro* assay for insulin (Waugh *et al.*, '50).

The insulin solution that transforms within 30 minutes at 80–100°C. may be quite stable at lower temperatures (e.g., 0–25°C.), the best being stable at pH 2.0 at temperatures in the latter range for at least months and possibly years. If, however, fibrils formed at higher temperatures (and cooled) are added to solutions of native insulin well within the range of stability, the fibrils thus seeded grow at the lower temperatures and remove the native insulin, transforming it into

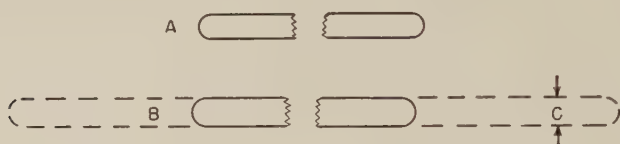


Fig. 1 Illustration of the growth of a preformed fibril, A, in the presence of a solution of native insulin. When growth is at a low temperature, the fibril at C consists of insulin that would have remained in solution for months in the absence of the seeding fibril.

fibrous insulin having properties similar to fibrous insulin formed at higher temperatures (Koltun *et al.*, '54). Thus in figure 1, the seeding fibril, A, grows as indicated in B. The insulin at C represents aggregated insulin that has not existed at a temperature expected to induce intrinsic instability, yet this insulin can combine with additional insulin to perpetuate the characteristic structure of the fibril at the lower temperature.

The rate of insulin removal, at lower temperatures, per milligram of seeding fibrils may be greatly increased by rapid freezing and thawing of the seeding suspension (Waugh *et al.*, '50), which breaks the original fibrils into segments. It is quite apparent that the breaks produce ends having an un-

usual affinity for native insulin, for the rate of insulin removal is considerably greater than that predicted on the basis of the fractional increase in total surface area. This and other evidence give reason for the belief that each segment grows in such a way as to restore a length consistent with the segment diameter; that is, as though there were a preferred axial ratio.

The over-all (i.e., high-temperature) and growth reactions of insulin fibril formation suggest that the initiation of a fibril (nucleation reaction) requires the near-simultaneous interaction of several monomers, the preparation of these monomers for bonding and the extent of approximation being temperature sensitive. Having been formed, the fibril nucleus and the resulting fibril present a surface configuration that is a composite of the properties of the individual molecules. Once the appropriate surface configuration is formed, an entering monomer finds itself in association with the requisite number of neighboring monomers to produce a stable linkage at either higher (e.g., 80°C.) or lower temperatures (e.g., 10°C.). On this basis, the rate of nucleation should vary with some power of the concentration greater than 2, and the rate of the growth reaction should be a function of the surface area of the fibril population and of the free insulin concentration.

Apparently, the cooperative effect represented by the appropriate surface configuration can operate only after a certain number of monomers have been linked in the correct spatial configuration, the smallest number being that required to give a stable nucleus. On this basis, nucleation and growth should be affected differently by changes in the environment. We can demonstrate that this is true by altering variables such as ionic strength and *pH*, the differences in effect on nucleation and growth in these cases being relatively small. Clear differential effects in nucleation and growth have been

described (Waugh *et al.*, '53) for solvents such as organic acids in high concentration in the absence of added salt (e.g., 8.3 *M* acetic acid), high concentrations of urea at *pH* 1.6 or 7.0 (e.g., 4.6–5.8 *M* urea), and aqueous phenol (e.g., 10–20% phenol). In these solvents, nucleation may be effectively absent but growth, although retarded, will proceed to completion (i.e., to the point where monomeric insulin is absent). It has been pointed out (Waugh *et al.*, '53) that a change in variable that introduces an increment, for example, in the energy barrier preventing close approach of molecules, will have its greatest effect on nucleation that requires the nearly simultaneous interaction of three or four monomeric units.

The cooperative effect may also give rise to a template on which new types of structure may originate. For example, as indicated, native insulin dissolved in 10% phenol at *pH* 1–2 does not show the nucleation reaction and, consequently, fibrils will not form from native insulin dissolved in this medium. Indeed, such a solvent (10% phenol, *pH* 1.6, 60°C.) will cause fibrils formed from native insulin in hydrochloric acid, termed FN, to revert to native insulin, for in 15 minutes over 95% of the insulin present may be recovered in a form that will crystallize. If, however, the time in phenol is extended to 0.5–2 hours, a fibrous system reappears. These new fibrils that form in the presence of phenol, designated FN-FN (P), are different from FN for, whereas FN are readily reverted by an alkaline treatment (Waugh, '48), FN-FN (P) are reverted by alkali only with difficulty, if at all. FN and FN-FN (P) are, however, structurally similar, since they give similar X-ray diffraction patterns (Koltun *et al.*, '54) and have similar appearances in electronmicrographs. It should be noted at this point that, whereas the electronmicrographs of FN show fibrils having relatively straight axes, the FN-FN (P) frequently have long axes that describe a helix of low pitch and are found twined around each other

in two's or three's. This same situation is often shown also in suspensions of fibrils obtained by seeding (unpublished).

Since fibrils do not form spontaneously from native insulin in aqueous phenol under conditions where they will form from FN, it seems reasonable to suppose that, although the set of FN are dissociating in phenol, surface regions will occur that, through a cooperative effect, lead to new types of nuclei, stable in the presence of phenol. It might be supposed that the reversion product obtained in aqueous phenol, although it crystallizes and has full biological activity, differs in some subtle way from native insulin. Denial of this is indicated by the behavior of mixtures of FN and native insulin. In aqueous phenol the FN disappear as expected, with the subsequent re-formation of fibrils. The native insulin present is incorporated in these fibrils.

From the time of maximum reversion of FN to the formation of a substantial set of FN-FN (P) is 10-15 minutes. Since maximum reversion leads to insulin monomer recoveries $> 95\%$, it is apparent that the quantity of insulin invested in nuclei stable in aqueous phenol is relatively small. The time period between maximum FN reversion and the formation of FN-FN (P) is thus a lag or latent period during which the nuclei formed from or on the preceding set of FN are growing to sizes that significantly decrease the amount of insulin free in the aqueous phenol solvent. We would expect the reaction leading to FN-FN (P) to take such a course from the characteristics of the formation of FN in solutions of native insulin in hydrochloric acid, for both appear to be based on linked nucleation and growth reactions.

THE OVER-ALL REACTION

The appearance of insulin fibrils has been examined in solutions containing 2.0, 1.0, and 0.5% insulin under conditions where the temperature, pH , and ionic strength are carefully controlled. Each curve of figure 2 represents two or three experiments. Experimental conditions were chosen that would

minimize the interfering effects of spherite formation (Waugh, '46) or other types of fibril aggregation. To obtain the data, we removed fibrils by ultracentrifugation and analyzed the supernatants for residual insulin by ultraviolet absorption.

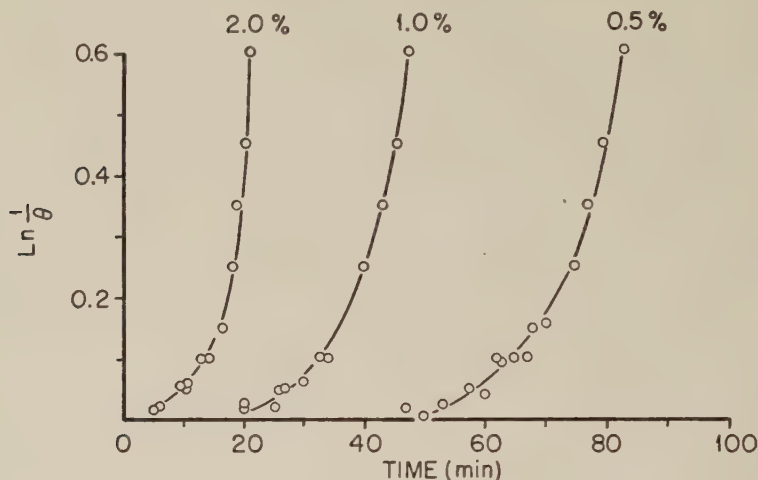


Fig. 2 The spontaneous transformation of native insulin into insulin fibrils is termed the over-all reaction. The three curves were obtained at 80.2°C., pH 1.55, and ionic strength 0.05. θ is the fraction of the initial insulin remaining free in solution.

Each reaction curve has a typical lag period during which little insulin appears in the fibrous form. There follow a nonlinear phase and a near-linear phase that, in purified insulin preparations, will extend to $\ln 1/\theta$ values in excess of 3.0 ($\theta = 0.05$).

NUCLEATION AND GROWTH

For stability, the nucleus requires the nearly simultaneous interaction of several monomers. Without defining closely the physical meaning of simultaneity, we should observe experimentally that nucleation can be represented by equation (1), where the rate of nucleation, dn/dt , is given by the product

of a rate constant, k_1 , and the protein concentration, C , raised to a power, p , that is the order of the nucleation reaction

$$\frac{dn}{dt} = k_1 C^p. \quad (1)$$

When more molecules are added to the nucleus, a particle (fibril) will eventually arise whose surface will have certain average properties. Let us assume that structures intermediate between that of the nucleus and the fibril having average surface properties may be neglected on the basis that the total surface presented by them will be small compared to the total surface of the fibril population. Any individual fibril can then be assumed to grow according to a rate constant, k_2 , the collision frequency per unit area of fibril surface (proportional to C), and the product of the surface area and the average condensation coefficient. The latter product will be termed the aggregating area, A . The condensation coefficient will include steric factors as well as factors such as charge repulsion. The growth of an individual fibril can then be described by equation (2).

$$\frac{dm}{dt} = k_2 AC \quad (2)$$

where m is the mass of the fibril. Essentially the same result will be obtained if molecules, having impinged and bonded at one locus, migrate over the fibril surface to a locus of lower energy.

If the average condensation coefficient does not change with the size of the fibril, we may relate the aggregating area, A , to the fibril mass. We may do this by assuming that the fibril is characterized by a constant axial ratio z regardless of size and that the fibril conforms to a model shape, for example, a spheroid, a cylinder that accepts molecules over its entire surface, or a cylinder that accepts molecules only at its ends. In all cases, the area may be obtained from the

axial ratio, z , and $m^{2/3}$ where m is the mass of the fibril. For a prolate spheroid the area is given by

$$A = \left(2\pi + 2\pi z \frac{\sin^{-1}e}{e} \right) \left(\frac{3m}{4\pi z \rho} \right)^{2/3} \quad (3)$$

where e is the eccentricity, z is the ratio of semimajor axis to semiminor axis, and ρ is the density of the fibril. For a spheroid of large asymmetry, $e \sim 1$. Neglecting 2π in the first factor leads to

$$A = 3.12 \times 10^{14} z^{1/3} m^{2/3}. \quad (3a)$$

When m is expressed in milligrams, A will appear in square Angstrom units.

Equation (2) may now be written:

$$+ \frac{dm}{dt} = k_2 m^{2/3} C. \quad (4)$$

We would expect to describe the course of the over-all reaction (nucleation and growth) by combining equations (1) and (4), using appropriate constants. Values of k_1 and k_3 were estimated from the numbers of fibrils present at the end of the over-all reaction and from the growth characteristics of seeded solutions. If C is expressed in milligrams of insulin per milliliter and m is in milligrams, the values are estimated to be approximately $k_1 = 1 \times 10^9$ and $k_3 = 1 \times 10^{-6}$. The value of p may be obtained from over-all reactions (fig. 2) carried out under carefully controlled conditions except that the insulin concentration is varied. The times necessary for conversion of a small chosen amount of insulin will then lead to the constant p . The nucleation and growth characteristics described at the start suggest that p will have a value greater than 2. Since a cooperative effect involving more than four or five neighbors seems unlikely for steric reasons, it is not surprising that the experimental value of p is between 3 and 4.

Equations (1) and (4) were integrated numerically by use of $p = 3$ or 4 and several paired values of k_1 and k_3 including those given. The details of the reaction are sensitive to the specific values chosen but all reactions share certain interest-

ing characteristics that may be illustrated by a single case; namely, $p = 3$, $k_1 = 1 \times 10^9$, $k_3 = 1 \times 10^{-6}$, and $C_0 = 20$ mg per ml, where C_0 is the initial insulin concentration.

Integration involves the mass of the stable nucleus. The nucleus mass was taken as 8.4×10^{-17} mg, which corresponds to 4 units of molecular weight 12,000. We chose this value with the following information in mind: (1) the rates of both fibril nucleation and growth decrease as the pH is increased above 2.0, the rate decreases paralleling the polymerizations of units of 12,000 that take place over the same pH range (Onley *et al.*, '52); (2) in solutions where a dissociation into monomers of 6000 has been demonstrated,¹ the rate of nucleation is decreased and may be negligible; and (3) the value of p is 3 or 4. Also, calculations show that the course of the reaction is not particularly sensitive to the mass of the stable nucleus.

This calculated reaction, calculation G, leads to a suspension containing 9.4×10^{13} fibrils per ml. It is instructive to examine both the final properties of the fibril population and the changes that occur as these properties are attained.

Curve A of figure 3, which is the fraction of the original insulin remaining in solution, records the disappearance of insulin with time (abscissa). A negligible change in θ is seen during the first 8 minutes, when $\theta = 0.95$, after which θ decreases rapidly and achieves a value of 0.1 in 22 minutes. The lag period so developed is evident in curve B, where the $\ln 1/\theta$ versus t plot of figure 3 is close to the corresponding reaction curve shown in figure 2. The lag period is followed by a relatively linear rise. Where the lag period is not so obvious we would be tempted to describe the reaction as first order and to subtract some time for "temperature equilibration," as has occasionally been done.

Curve C of figure 3 gives, for each successive time unit, the number of fibrils formed during this time interval. It is seen that the number of fibrils formed is relatively constant

¹ These data will be published in collaboration with Dr. David A. Yphantis.

at 8×10^{12} per minute for the first 8 minutes, after which N drops rapidly to small values. However, even at 16 minutes, appreciable numbers of nuclei are being formed. Nevertheless, of the total number of fibrils formed, 65% are in existence when $\theta = 0.95$; that is, they are formed during the lag period of the first 8 minutes.

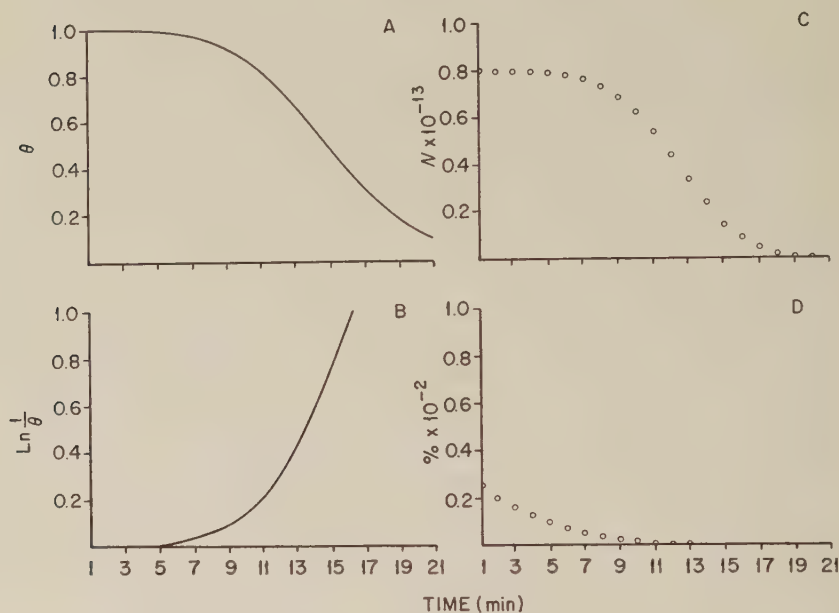


Fig. 3 Calculation of reaction characteristics for $k_1 = 1 \times 10^9$, $k_2 = 1 \times 10^{-6}$ and $p = 3$. Curve A represents the fraction of initial insulin remaining free in solution at time t . Curve B represents the course of the reaction. Curve C represents the number of fibril nuclei that form in each time interval and curve D represents the percentage of the fibrous insulin accounted for by each group of nuclei when $\theta = 0.1$.

Curve D of figure 3 refers to conditions when $\theta = 0.1$, that is, after 22 minutes reaction time. Each circle gives the percentage of the fibrous insulin represented by that particular group of fibrils. Thus the fibrils whose nuclei are formed during the first minute will remove 25% of the insulin; whereas those formed during the eighth minute will remove only 3.1%

of the insulin. By summation it is clear that the fibrils formed during the lag period will remove over 93% of the total insulin. As seen in the preceding paragraph, these constitute 65% of the total. Thus it is apparent that the subsequent course of the reaction is essentially established when $\theta = 0.95$. Some of the fibrils formed after the first 8 minutes should be visible, for example, in electronmicrographs, although most are cal-

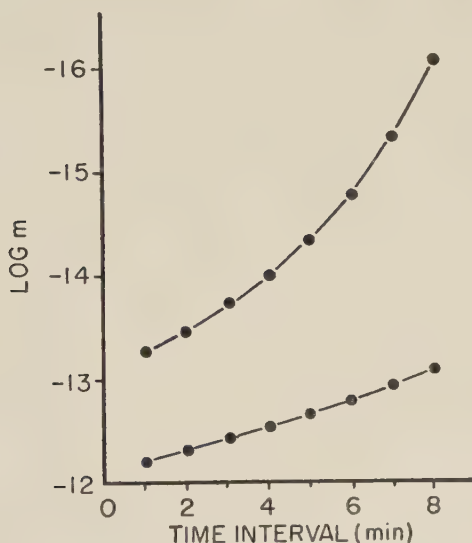


Fig. 4 Mass distribution of fibrils that form during first 8 minutes reaction time. Upper curve, mass distribution at 8 minutes, and $\theta = 0.95$, and lower curve, mass distribution when $\theta = 0.1$ (22 minutes).

culated to be not visible. Since the reaction is dominated by the fibrils formed during the first 8 minutes, these will be referred to as the significant fibrils.

Figure 4 shows the distribution of masses among the significant fibrils. It should be remembered (fig. 3) that the numbers in each group are approximately constant at 8×10^{12} since they vary only from 8.0 to 7.36×10^{12} , the average being 7.75×10^{12} fibrils per group. Figure 4, upper curve, gives the distribution of individual masses at 8 minutes reaction time. Here $\log m$ (where m is in milligrams) is plotted

versus the time interval during which these fibrils formed. At 8 minutes reaction time, the largest fibril of the significant group is 640 times the mass of the smallest, which at this time is a stable nucleus. After the reaction has progressed to the point where $\theta = 0.1$ (22 minutes) the mass of the largest is now only 7.4 times that of the smallest. This striking convergence of mass is due to the fact that growth is proportional to a surface property, which in turn will be proportional to $m^{2/3}$.

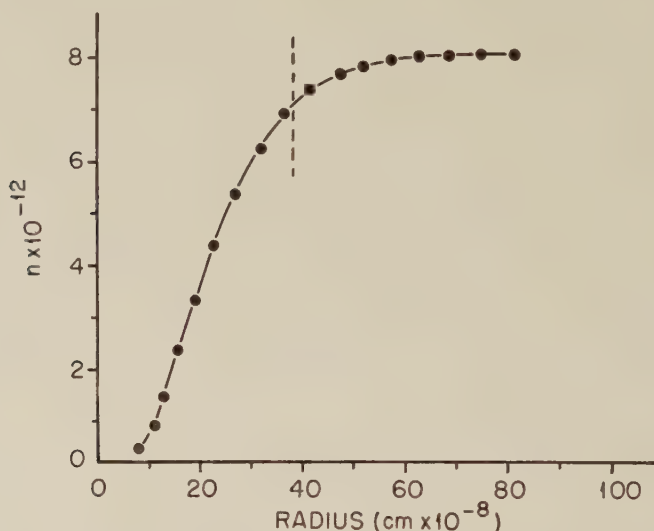


Fig. 5 Distribution of numbers of fibrils (when $\theta = 0.1$) with respect to the radius corresponding to each group.

If the radii of the fibrils are examined, a property more easily tabulated than fibril length in visual observations, a similar situation is seen. Figure 5 is a plot of the radii of the individual fibrils (in centimeters) versus the number of fibrils having each radius. The distribution of radii has a sharp upper limit, corresponding to the radius ($\sim 82 \text{ \AA}$) of the fibrils first formed. There follow both in order of decreasing size and time of nucleation seven additional values, corresponding to the group of significant fibrils. This group, which

as stated comprises 65% of the total number of fibrils and removes 93% of the insulin, has a distribution in which the largest radius is 1.95 times that of the smallest. An appropriate sampling technique would reveal, however, significant numbers of fibrils having diameters that decrease progres-

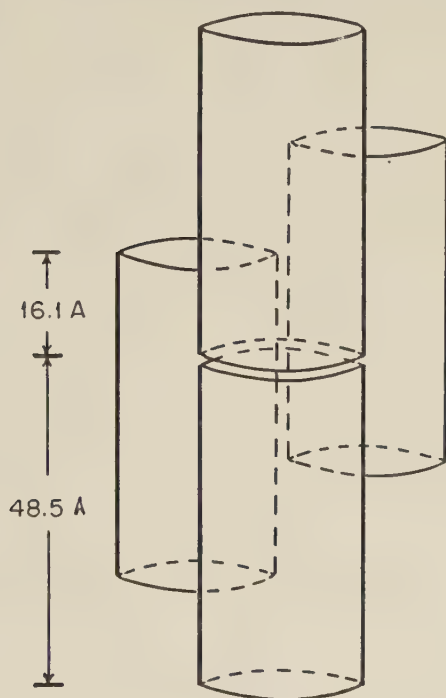


Fig. 6 Schematic representation of the stable insulin fibril nucleus.

sively, certainly to the limit of resolution of present techniques that allow direct observation.

Some of the calculated fibril radii are close to the radius of the insulin molecule and some, if calculated, would be much smaller. This is a consequence of the assumption of a constant axial ratio. As pointed out, there must be a transition between the stable nucleus depicted in figure 6, based in part on evidence for the X-ray diffraction patterns of fibril (Koltun *et al.*, '54), and the fibrils of more average axial ratio.

GROWTH

Whereas the nucleation reaction cannot be examined in the absence of growth, the growth reaction alone may be conveniently examined when preformed fibrils or fibril segments are seeded into native insulin at temperatures where nucleation is absent during the observation period. A single fibril

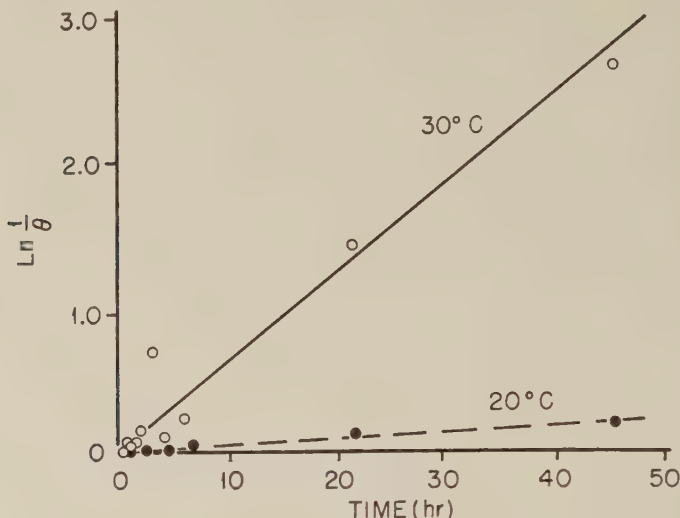


Fig. 7 Fibril growth at temperatures of 20° and 30°C. Reaction solutions contained 1.8% native insulin and 0.1% of seeding fibrils prepared by freezing and thawing a standard insulin gel. The reaction was carried out at pH 1.65.

having a mass m_s , when seeded into a solution of native insulin, will grow according to

$$\frac{dm}{dt} = k_s (m_s + m) \% C \quad (5)$$

where m is the mass added during growth. If the seeding fibril is normal, i.e., if it has a mass and dimensions corresponding to those it would achieve normally during fibril formation, we would assume that m_s would be given by the actual mass of the fibril. But m_s may not represent the actual mass of the seeding fibril; e.g., if a long fibril is broken into segments, each segment will have a diameter disproportionately large compared with its length. The effective aggregating area may then approximate the aggregating area of the original fibril. The fibril

segment may then elongate with constant aggregating area until its normal axial ratio is achieved, after which further growth will be accompanied by an increase in both length and diameter and consequently with an increasing aggregating area. If enough seeding segments are used, all of the native insulin will disappear before any increase in aggregating area occurs. In this case, the growth characteristics of a population of equivalent fibril segments will be given by

$$\frac{dm}{dt} = -\frac{dC}{dt} = n k_s A_s C \quad (6)$$

where n is the number of segments and A_s is the aggregating area of each segment. Growth should then appear as a first-order reaction, namely,

$$\ln \frac{C_0}{C} = \ln \frac{1}{\theta} = n k_s A_s t. \quad (7)$$

Such a situation is shown in figure 7 for two different temperatures.

When normal fibrils are used as seeds the course of the growth reaction will depend on the mass of seeding fibrils relative to the amount of native insulin available. We may examine the situation by assuming a uniform population of seeding fibrils, an assumption that is consistent with the properties of the fibril population at the end of an over-all reaction. For a uniform population of fibrils used as seed, $n m_s = C_0 - C$. Substitution in equation (5) gives

$$-\frac{dC}{dt} = n^{1/3} k_s (n m_s + C_0 - C)^{2/3} C. \quad (8)$$

We may now examine the effect of varying the amount of seed by choosing representative values of $n m_s$ in terms of C_0 . Thus let $n m_s = \phi C_0$ and $B = 1 + \phi$. Equation (8) then gives, on integration,

$$n^{1/3} k_s t = \frac{1}{2(BC_0)^{1/3}} \ln \frac{B^{2/3} + B^{1/3} (B - \theta)^{1/3} + (B - \theta)^{2/3}}{B^{2/3} - 2B^{1/3} (B - \theta)^{1/3} + (B - \theta)^{2/3}} - \frac{\sqrt{3}}{(BC_0)^{2/3}} \tan^{-1} \frac{\sqrt{3} B^{1/3}}{B^{1/3} + 2(B - \theta)^{1/3}} + \text{constant}. \quad (9)$$

We evaluate the constant of integration by noting that at zero time $C = C_0$ and $\theta = 1.0$. Figure 8 shows the effect of varying

ϕ . When the amount of seeding fibrils is comparable to the amount of free insulin, the free insulin disappears before a significant increase in aggregating area occurs and the reaction, plotted as a first-order reaction, will give a nearly straight line as indicated for $\phi = 0.5$ and $C_0 = 20$ or 10 mg per milliliter. When ϕ is smaller, the reaction curves have a pronounced upward curvature as indicated for $\phi = 0.02$ and $C_0 = 20$ or 10 mg per milliliter. The upward curvature is caused by the increase of the aggregating area with time.

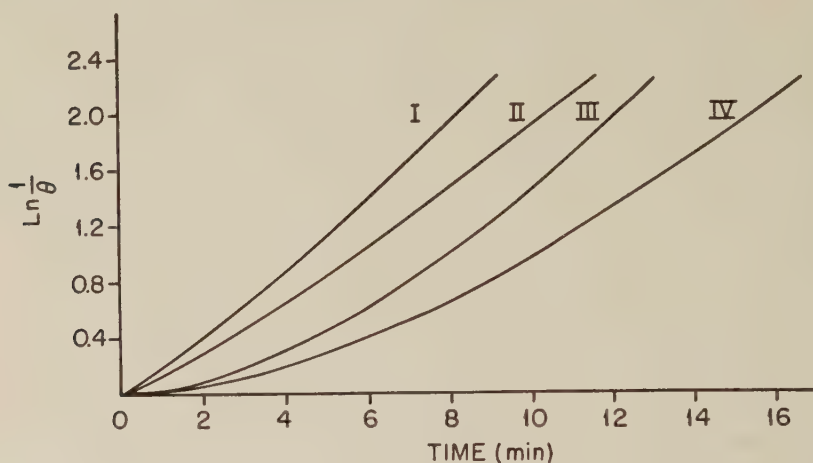


Fig. 8 Fibril growth curves calculated for different relative amounts of normal seeding fibrils, using equation (9). Curve I: $C_0 = 20$; $\phi = 0.5$. Curve II: $C_0 = 10$; $\phi = 0.5$. Curve III: $C_0 = 20$; $\phi = 0.02$. Curve IV: $C_0 = 10$; $\phi = 0.02$.

Figure 9 shows curves from experiments carried out at two levels of seeding insulin. The transition from a near-linear relation between $\ln 1/\theta$ and t to a curve whose slope increases with t is evident. The curvature in the experimental plots is somewhat greater than expected on the basis of the relative amounts of seeding material used, since $\phi = 0.25$ for 0.5% seeds and $\phi = 0.06$ for 0.125% seeds. However, the relative initial reaction velocities suggest that ϕ is smaller than 0.06 in the second case. The seeded solutions in the two cases are different, for the fibril suspension is more stable at the higher seeding concentration. At the lower seeding concentration, some of the fibrils aggregate, occasionally to

form spherocrystalline structures similar to those that have been previously described (Vaugh, '46). Spherite formation effectively removes fibrils from suspension and thus interferes in such a way as to decrease the value of ϕ . The effects of spherite formation, or other types of aggregation, and possible fragmentation make difficult a more quantitative treatment of the growth curves so far obtained.

There can be little doubt that the development of insulin fibrils is dependent on nucleation and growth reactions whose relative rate constants, along with the initial insulin concentration, determine the distribution of the resulting fibril

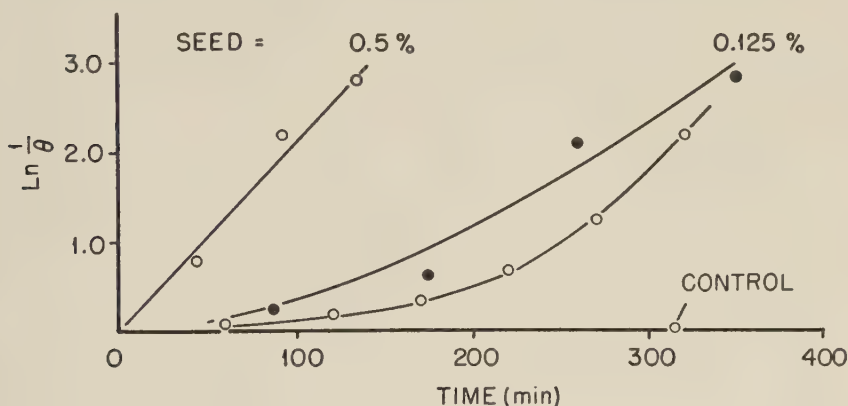


Fig. 9 Fibril growth curves obtained at 50.3°C., pH 1.50, and ionic strength 0.05 for 2% native insulin seeded as indicated. Two experiments were performed at 0.125% seed.

population. A significant relative change in the values of the constants leads to striking alterations in the fibril population. For example, to avoid spherite formation, we carried out the reactions described so far at a low ionic strength, usually 0.05. If the ionic strength is increased, the rate constants for nucleation and growth increase but that for nucleation increases more rapidly. The result is a population of numerous short fibrils that have a strong tendency to aggregate into spherites. On the other hand, the rate of nucleation decreases rapidly with temperature. Thus, at temperatures near 50°C., a few fibrils form and grow to

enormous size. The same effect occurs as the insulin concentration is decreased. The rate of nucleation, varying with the free insulin concentration raised to the third or fourth power, can be made slow compared to the growth rate. Again, a few large fibrils are formed.

BIOLOGICAL SYSTEMS

The uniformity of fibrils observed in biological preparations may have its origins in controlled nucleation and growth reactions similar to those described here for insulin. The rapidity of the fibril-forming reactions would not affect the end result, even if they were much faster or slower than those described for insulin, for diffusion is not a limiting factor in insulin fibril reactions that take more than a few minutes to go to completion.

In certain biological reactions, such as the conversion of fibrinogen to fibrin, nucleation and growth reactions appear to play an important role in clot development. For example, the coarseness or fineness of a clot can be controlled by controlling the rate at which "activated fibrinogen" is formed. Generally, when the activation reaction is rapid a fine clot results and, conversely, when activation is slow the clot is coarse. This is what would be expected; for a fast activation should lead to a momentary high concentration of activated fibrinogen, which in turn should produce many nuclei for fibrin strand formation. The situation is expected to be complicated, however, for it is likely that fibrin strand formation also involves the incorporation of smaller polymers in the formation of larger polymers.

Calculations suggest that, in insulin solutions, the nuclei that form first dominate the reaction. From this it would appear that the most effective control in *in vivo* systems would be through a control of both the time interval during which nuclei are formed and the rate of nucleation during this interval. Such a control might be attained directly, for example, through the action of a molecule that acts as a nucleus template, as do FN evidently in the formation of FN-FN (P), or indirectly through the action of an enzyme

that modifies certain substrate molecules so that they form nuclei more easily, the remainder of the substrate molecules aggregating without modification onto these nuclei. Considerations such as these do not of course do more than indicate possible mechanisms and suggest directions that may prove fruitful in approaching the problems of fibrinogenesis *in vivo*.

GENERAL DISCUSSION

FERRY²: I think I will wait until after Professor Flory's paper, on which I have already planned to say something.

I would like to ask a question, however, if I may, as to whether the distribution of lengths and diameters, which I gather you predicted from your theory, has been compared with experimental count.

WAUGH: A careful examination of diameter and length distributions has not yet been made. We have compared diameters of large fibrils in electronmicrographs but lengths cannot be determined easily since it is difficult to see the entire fibril in a single electronmicrograph.

Information about average lengths may be obtained from flow double refraction. These have been used to observe the effects of alterations in the nucleation rate constant relative to the growth rate constant. For example, if the insulin concentration is decreased, the nucleation rate is decreased much more rapidly than the growth rate. The result is a population of a few enormous fibrils. If salt is added, the nucleation rate is increased more pronouncedly than the growth rate; so that in the presence of salt we find a population consisting of a tremendous number of small fibrils.

RANDALL³: I would like to express my admiration of this very excellent work that Dr. Waugh has given. No doubt for lack of time, he has not told us in what way the polypeptide chains in the globular form change into the fibrous form. Has he any comments at all as to whether these chains unfold in transferring from one form to the other and any ideas of what that mechanism is?

² John D. Ferry, University of Wisconsin.

³ J. T. Randall, University of London King's College.

My second point is a more technical one. You said that you looked at the X-ray photographs and you could see two distinct forms of fibrils. I would just like to ask if the photographs were taken on really wet material or whether it had been allowed to dry in any way.

WAUGH: The answer to the first question is that the insulin molecule does not unfold in forming the fibril. One can carry out, for example, the growth reaction at close to 0°C. Under these conditions, since native insulin is quite stable in acid (indeed a *pH* of 2 is its *pH* of maximum stability), I can see no reason why the molecule should unfold.

Another piece of evidence in this direction is that a system of fibrils can be reverted quantitatively to give crystalline, biologically active insulin. Indeed, the formation and reversing of fibrils is a nice way to purify insulin. Fibril formation is a highly specific reaction on the part of insulin.

RANDALL: Should not the X-ray photographs in the two forms then be essentially the same?

WAUGH: They are. Essentially all the fibrils we have studied give the same diffraction pattern.

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THE FIBROUS PROTEIN OF THE NERVE AXON¹

FRANCIS O. SCHMITT

Biology Department, Massachusetts Institute of Technology, Cambridge

ONE FIGURE

Since the earliest cytological investigations of nervous tissue, attention has been focused on the fibrous constituent of the neuron, which is seen most clearly in the axoplasm of peripheral nerve fibers in the form of neurofibrils. This fibrous constituent is characteristic of nerve fibers; it appears to be demonstrable in all types of nerves when appropriate histological methods are employed. It would seem therefore that it must serve some characteristic, possibly vital, function in the neuron. Many speculations have been offered (see the reviews of Peterfi, '29; Parker, '29; Gerard, '31) but at present we still have no clear knowledge of their function.

Several reasons for the apparent lack of progress in the investigation of the structure, composition, and function of this fibrous protein may be mentioned. Chief among these is the idea, which became quite pronounced among physiologists a generation ago, that neurofibrils are artifacts of histological preparative technique rather than components existing in the axoplasm of normal, functioning nerve fibers. The axis cylinders of most freshly dissected nerve fibers appear to be devoid of fibrous structure when viewed with the light microscope (including the polarizing and ultraviolet microscopes). Only after chemical alteration or histological

¹These studies were aided by a research grant (B-24) from the National Institute of Neurological Diseases and Blindness, of the National Institutes of Health, U. S. Public Health Service; by a contract between the Office of Naval Research, Department of the Navy, and the Massachusetts Institute of Technology (NR 119-100); and by a grant from the Trustees under the wills of Charles A. King and Marjorie King.

treatment do the fibrils make their appearance. Another reason is that it is difficult to isolate the fibrous material from nervous tissue and to characterize it physically and chemically. The fibrous protein of the axon represents but a very small fraction of the solids of axoplasm and a still smaller fraction of whole nervous tissue such as peripheral nerve, spinal cord, or brain. Moreover, almost no information is at hand by which one might identify the axon protein in any purified fraction of nerve tissue. In this respect the situation is very different from that of muscle, where the contractile protein represents a substantial fraction of the substance of the myofibril and can readily be prepared from minced muscle.

The only material from which the fibrous protein may be obtained uncontaminated by sheath and extracellular constituents is the axoplasm from squid giant fibers. Accordingly, the isolation and physicochemical characterization of the axon protein has been made one of the main aims of our own "squid operations" at M.I.T. during the last 8 years. In this communication, I shall review briefly the results thus far obtained in this investigation and indicate the lines along which further progress may be expected.

STRUCTURAL CHARACTERIZATION OF THE FIBROUS PROTEIN OF THE AXON

Polarization optical analysis (Bear *et al.*, '37) indicated that the fibrous protein of the squid axon occupies but a small fraction of the axon volume (ca. 0.07%) but has an intrinsic birefringence comparable to that of other typical fibrous proteins (ca. 0.005). These results are consistent with the electron microscopic evidence, obtained later, that reveals the protein as clearly defined filaments that occupy a small portion of the axon volume.

Early efforts to obtain a characteristic X-ray diffraction fiber pattern from squid axoplasm dehydrated in alcohol were unsuccessful (Schmitt *et al.*, '39). Subsequently, efforts were made from time to time by various methods of dehydration and orientation but these were likewise unsuccessful.

However, more recently Dr. Carolyn Cohen, in collaboration with Dr. Betty Gerén Uzman, succeeded in obtaining very thin fibers from squid axoplasm. The axoplasm, dialyzed free of salts, was concentrated to a gel by ultracentrifugation. In several instances an oriented fiber pattern was obtained resembling the typical alpha pattern of fibrous proteins of the KMEF class. The spacings of the reflections were 5.1 Å on the meridian and 9.6 Å on the equator. These results must be considered provisional. If they are confirmed by experiments now in progress, they will provide the first evidence about the polypeptide chain configuration of the nerve protein.

In the electron microscope the fibrous protein appears as filaments 100–200 Å wide and indefinitely long. They have been studied not only as obtained from solutions of axoplasm (Schmitt, '50; Schmitt and Geren, '50; Fernandez-Moran, '54) but also in thin sections of various kinds of nerve fibers (see Fernandez-Moran, '54, for a review). The thin, smooth axon filaments are associated with small, dense granular material that gives them a nodose appearance and suggests an axial periodicity in the neighborhood of 250 Å. The spacing between granules is however not uniform even in individual filaments. Moreover, in purified preparations the filaments frequently appear to be quite smooth. It seems probable, therefore, that the granular material, though tending readily to complex with the fibrous protein is actually either a different substance or is in a quite different, perhaps highly folded, configuration.

In longitudinal thin sections, the axon filaments are seen as indefinitely long threads having an essentially parallel disposition through the axon and passing without interruption through the nodes of Ranvier. The neurofibrils of classical histology represent aggregates of axon filaments large enough in cross section to be resolvable in the light microscope; deposition of heavy metal, as with the histological silver and gold techniques, also favors detection of very thin fibrous structures by the light microscope.

VISCOSITY OF SOLUTIONS OF AXOPLASM

Unpublished observations made by A. H. Hodge and M. A. Jakus at the Marine Biological Laboratory at Woods Hole in the summers of 1951 and 1952 indicated that when squid axoplasm is dispersed in cold distilled water, *pH* 6-7, the relative viscosity is quite high. Elevation of the ionic strength by addition of KCl reduces the viscosity; at ionic strengths of about 0.15 the viscosity is not greater than that of the solvent. Dialysis against water raises the viscosity significantly though seldom to the full original value. B. B. Geren (also unpublished) studied this type of behavior in more detail in the summer of 1952. The relative viscosity of axoplasmic solutions or dispersions in water or in glycine solution proved to be quite high and to remain high for many hours, depending on the history and characteristics of each preparation. These preparations were first subjected to centrifugation at about $25,000 \times g$ to remove the larger particulates and tissue fragments. It is difficult to separate mitochondria and the smaller particulates by simple centrifugation because the filaments cause the preparation to come down as a gelled mass. Probably because of a slow denaturation, the viscosity falls when such solutions are stored, even though kept cold. Viscosities of preparations obtained by dialysis against water or glycine, after previous elevation of the ionic strength by the addition of salts, cannot exceed this slowly receding upper limit.

These experiments on solutions or dispersions of whole unfractionated axoplasm supported the idea that the viscosity of such preparations is caused by the presence of the long filaments of axon protein and that, subject to certain irreversible processes of denaturation, these long strands are broken up by elevation of ionic strength into smaller particles, possibly the protein molecules; by reversible polymerization these can again form the long, filamentous strands. Under conditions prevailing in the normal axon, the system may be poised so that a change of ionic environment may push

the system in the direction either of aggregation or disaggregation of the fibrous axonal protein. It may be appropriate to point out here that the fibrous protein is not necessarily limited to the axon filaments that course through the substance of the axoplasm but may also be deposited upon the limiting envelope of the axon, the axolemma. If this should prove to be true, the ultrastructure of the surface membrane of the axon may be very responsive to change in ionic environment. Increase in ionic strength in highly localized regions, such as is thought to occur during the phases of the action wave, would lead to a "depolymerization" or disaggregation of the protein. Whether such changes occur, and are important in the changes in permeability of the "excitable membrane" during impulse propagation is as yet unknown.

Much of the further advance in our knowledge of the properties of the fibrous protein of the axon was accomplished by Maxfield ('51, '53), assisted in later experiments by R. W. Hartley. Their conclusions, based on observations of the properties of purified fibrous protein and to be summarized briefly, confirm the ideas just described, which were based primarily on studies of the viscosity of suspensions of whole axoplasm.

Fractionation by low-temperature alcohol treatment and by heavy-metal precipitation were attempted but Maxfield eventually adopted a very simple method of purifying the fibrous protein, as follows: Extruded axoplasm is taken up in phosphate buffer, $pH = 6.0$, $\mu = 0.1$. Undissolved material is removed by centrifugation and the remaining solution is subjected to differential centrifugation until the peak in the ultracentrifuge indicates no impurities. Such a preparation contains about 0.02% protein. When examined in the electron microscope the filaments are clean; relatively little amorphous material appears to be present (see fig. 1). The protein of the axon filaments was estimated to represent about 10% of the total macromolecular content. If Koechlin's estimate ('55) is assumed to be correct, this would amount to about 0.35%

of the wet weight of axoplasm or about 2.6% of the dry weight. The isoelectric point of the protein is close to pH 6.0.

Purified protein that has been sedimented in the ultracentrifuge is refractory to dissolution in phosphate buffer but is readily soluble in 0.1 M glycine or alanine solution. It was usually stored in such buffers in the cold.

From studies of specific viscosity in 0.1 M glycine solution, Maxfield deduced that the axial ratio of the macromolecule is very high (about 100). Simple dilution without change of ionic strength or pH causes the axial ratio to diminish



Fig. 1 Electron micrograph of purified axon filaments obtained from squid axoplasm as follows: The heavier particulate material was spun down at $25,000 \times g$, after which the supernate was spun at about $140,000 \times g$ for about 8 hours. The sediment was resuspended in 0.1 M glycine solution, $pH \cong 6.5$. Stained with phosphotungstic acid and shadowed with chromium. $\times 18,000$.

markedly, suggesting that the bonds between the molecules that compose the filaments are relatively weak. At $\mu=0.1$, the viscosity is much lower but still appreciable. At $\mu=0.1$, pH 8.5, the axial ratio is very low.

From light-scattering studies in glycine at pH 6.0, Maxfield concluded that the protein occurs as a flexible rod rather than as a random coil. The molecular weight was deduced to be about 10^8 . In phosphate buffer, pH = 8.5, $\mu=0.1$, the points on the Zimm plot were considerably higher than those for a solution at pH 6.0. The molecular weight under these conditions was estimated at 70,000. This suggests that as the pH and ionic strength are elevated the filaments decompose into globular molecules of about 70,000 molecular weight. It has been impossible as yet to obtain enough of this purified material to characterize it properly with respect to its amino acid composition and physical properties.

Maxfield and Hartley ('56) have demonstrated an apparent change in axon filament cross section as a function of pH. They have consistently observed that the sedimentation velocity of axon filaments in phosphate buffer is more rapid at pH 6.0 than at pH 7.7. Evaluation of the sedimentation constants by extrapolation was not feasible because of the variable viscosities of different preparations and because of their relative instability. Therefore, to gain insight into the effect of pH elevation, they prepared purified filaments and divided them into two parts, one of which was brought to pH 6.0 and the other to pH 7.7 (both at $\mu=0.1$). Viscosities were measured simultaneously on both aliquots and found to be identical (or possibly slightly higher at pH 7.7). These aliquots were then examined in the ultracentrifuge. The preparation at pH 6.0 sedimented markedly faster than that at pH 7.7.

These results suggest that the filaments are much narrower at pH 7.7 than at pH 6.0. The process is reversible; on passing from pH 7.7 to 6.0 the apparent width of the filaments again increases. The reversibility of the reaction was more clearly

demonstrated when a single preparation was examined in the ultracentrifuge consecutively at *pH* 6.0, then at *pH* 7.7, and finally again at *pH* 6.0. The filaments are much less stable at *pH* 7.7 and are less stable in pure solution than in whole axoplasm. This instability may cause protein to be lost, in studies of the reversibility of the changes that occur at constant ionic strength but changing *pH*.

Although the evidence points strongly to the idea that the diameter of the axon filaments is reduced when the *pH* is increased from 6.0 to 7.7, the mechanism of the change is unknown. The reduction in diameter may be caused by a splitting of the axon filaments into two components (which may be identical or different) or to a desorption of axoplasmic constituents from the fibrous protein (which seems to be highly charged). The change seems to be reversible and may prove to be of physiological significance because it occurs under conditions that are not very different from those obtained in normal axoplasm (the *pH* of extruded axoplasm is about 6.4).

We have not been able to obtain enough axoplasm to permit a determination of the amino acid composition of the purified fibrous protein. Nor could we determine which, if any, enzymes are intimately associated with the protein, although we are still trying to determine the enzyme content of whole axoplasm and of the Schwann cell sheath. When information about enzyme distribution is available, the biochemical and physiological role of the fibrous axon protein may become more evident.

The foregoing discussion emphasizes the need for more of the squid nerve material if further physicochemical and biochemical investigations of this protein, and the other individual constituents of peripheral nerve fibers, are to be successful. Since it is highly desirable that the material be available for longer than the few summer months, another source is being investigated. If appropriate arrangements can be made, we may be able to make use of the large squid that abound in the waters off the coasts of Chile and Peru.

GENERAL DISCUSSION

OSTER²: Why can't you just take an X-ray picture of the whole nerve itself?

SCHMITT: X-ray diffractions first obtained from whole nerve in collaboration with Drs. G. L. Clark and J. N. Mrgudich were at first attributed to the axon fibrous protein. However, subsequent investigation indicated that all the diffractions of whole nerve were caused by either the myelin or by the connective tissue. Failure of the axon fibrous protein to yield diffractions is probably due to its low concentration and rather low degree of orientation in the axoplasm of fresh nerve fibers. Even when the axoplasm is removed from the squid giant fibers and prepared under conditions that manifest a high positive birefringence, the orientation of the fibrous protein is still insufficient to make itself evident in the diffraction pattern; only two rings at about 4.5 and 9.6 Å are observed. Doctor Cohen now seems to have succeeded in inducing enough orientation to yield a fiber-type X-ray pattern.

FERRY³: I might mention an alternative method of analyzing light-scattering data for very long rods that was developed by Dr. E. F. Casassa ('55) in our laboratory. The Zimm plot does not give a very good estimate of either molecular weight or molecular length. The alternative method of plotting does, however, give a very good estimate of *width*. This might be of interest in connection with your splitting.

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² Gerald Oster, Polytechnic Institute of Brooklyn.

³ John D. Ferry, University of Wisconsin.

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ADDENDUM

During May and June large squid were obtained off Iquique and Viña del Mar, Chile, and the giant fiber system studied. From this experience it has become clear that these large squid can readily provide the source of the greatly increased amounts of axoplasm needed in our program of biophysical and biochemical nerve research. As much as 35 mg (dry weight) of axoplasm was harvested from the giant fibers of one relatively small (60 cm mantle length) squid. Efforts are now being made to arrange a practical program of squid collection and dissection and means by which relatively large quantities of material can be made available for physical and chemical analyses. We are greatly indebted to Mr. and Mrs. Louis E. Marron for their encouragement and active personal assistance in the expedition and for their generous financial assistance. We are also greatly indebted to Dr. Mario Luxoro for his invaluable assistance in this first phase of the "large squid operations."

CRYSTALLINITY AND DIMENSIONAL CHANGES IN FIBROUS PROTEINS

P. J. FLORY

Department of Chemistry, Cornell University, Ithaca, New York

THREE FIGURES

It is widely accepted that native proteins are crystalline, their peptide units being arranged in regular patterns. Raising the temperature or placing the protein in a suitable solvent destroys this regularity. The polypeptide chains then assume irregular, or random, configurations. The resulting transformed ("denatured") proteins exhibit elastic properties characteristic of a polymer in the rubbery state; i.e., in this state the protein behaves as a typical amorphous long-chain polymer.

The transformation from the crystalline to the amorphous state obviously is analogous to the melting of a simple crystalline substance. Latent changes in heat and volume may be observed in furtherance of this analogy. The question of whether such a process can be treated as a phase transition remains — more particularly, whether it can be characterized by a melting point, and whether thermodynamic theory can be applied to the transition.

An abundance of evidence on other polymers, including polyethylene, synthetic polyamides, synthetic polyesters, natural rubber, gutta-percha, and cellulose derivatives gives affirmative answers to these questions. Sharp melting points are observed on slow melting; depressions of the melting points by diluents, or by structural impurities, have been shown to be in accord with thermodynamic theory over wide ranges in both the amounts and the nature of the diluents, or structural impurities.

It has long been known that collagen and resting muscle exhibit increases in volume and absorption of heat upon contraction. The correspondence of the structural transformation that occurs during contraction to a melting process was recognized by Wöhlisch ('32, '39, '40, '43) and a few other early investigators. The significance of these observations does not seem to be fully appreciated by more-recent investigators.

We have undertaken the investigation of the volume changes in collagen-ethylene glycol mixtures as the temperature is raised slowly (Garrett and Flory, '56, and unpublished). A latent volume change is discernible, and it occurs over a narrow temperature range, the transformation being completed at a sharply defined temperature — T_m , the melting point characteristic of the mixture. When the sample is cooled and held at about 20°C. below T_m , a gradual decrease in volume sets in. This is indicative of recrystallization. Reheating causes a latent volume change at about the same T_m .

Figure 1 shows melting points determined in this manner by Garrett and Flory ('56, and unpublished) plotted against the volume fraction of collagen in the mixture. Also included are several melting points determined for more dilute mixtures by use of the polarizing microscope. Results at the lowest concentration (X) represent an observation of the temperature at which the viscosity of the solution decreased abruptly (Matthews *et al.*, '54; Gallop, '55; Boedtker and Doty, '56). The curve has been calculated from the standard melting point relation (Flory, '53)

$$1/T_m - 1/T_m^\circ = (E/\Delta H_u) (v_u/v_1) (v_1 - \chi_1 v_1^2) \quad (1)$$

where the melting point T_m° of the pure polymer is taken as 423°K., the heat of fusion $\Delta H_u = 2400$ cal per mole of structural units, the molar volume ratio v_u/v_1 of unit and solvent is 1.23, and the interaction parameter $\chi_1 = 150/T$ cal cc⁻¹deg⁻¹. The value chosen for the interaction parameter receives independent support from light-scattering and

osmotic measurements. The value for the heat of fusion is reasonable though somewhat larger than Wöhlisch's calorimetric value, 1200 calories per peptide unit, which, however, was determined in water.

Apart from a minor divergence at low concentrations, the entire phase diagram can probably be described by a single functional relation of the form applicable to other polymers. The shrinkage of collagen and the transformation of a dilute dispersion of rod-like protocollagen particles are thus shown

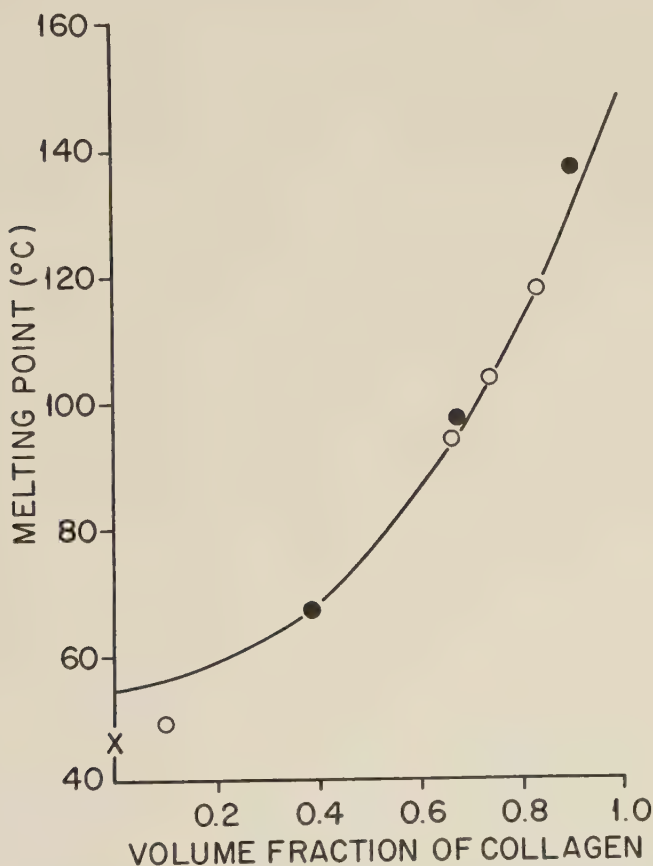


Fig. 1 Melting temperatures for collagen-ethylene glycol mixtures (Garrett and Flory, '56, and unpublished). Values determined dilatometrically (○), by disappearance of optical depolarization (●), and viscometrically (X).

to be one and the same phenomenon. Irrespective of whether the collagen occurs as relatively concentrated fibrillar aggregates or as a dilute dispersion, the underlying process consists in the destruction of the regular chain arrangement characteristic of crystalline (native) collagen.

The processes here considered should preferably be represented in two steps shown schematically in figure 2. The concentration of protofibrils released in step (I) from the

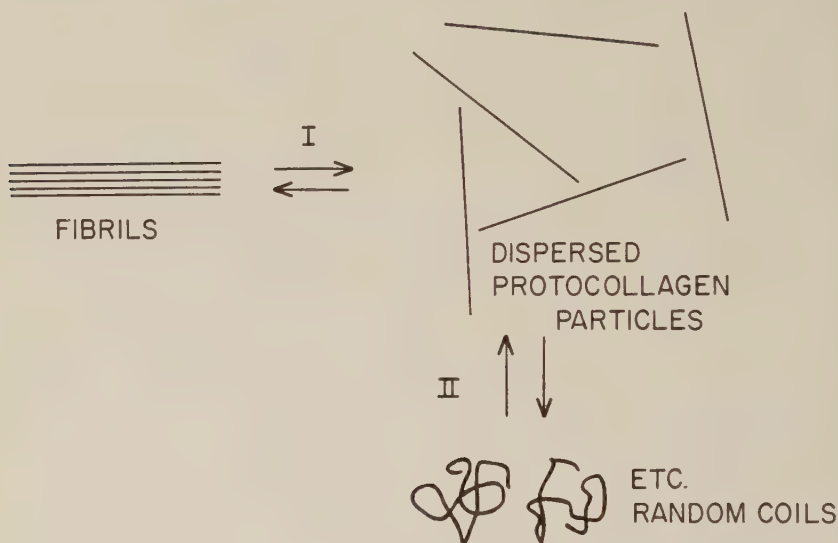


Figure 2

larger fibrillar aggregates (fibrils) could not under any circumstances be large. This statement can be justified by consideration of the high asymmetry of the protocollagen particles, whose axis ratio is about 200:1. The disordered arrangement of such highly symmetric particles in a solution becomes impossible above a concentration of about 5% (Flory, '56a). With the aid of only a small attractive energy between particles, the equilibrium (I) will be shifted strongly to the left. Although this energy will be much less than the energy change for process (II), it may nevertheless have a very

significant influence in suppressing the extent of dissociation of the primary fibrils according to process (I). This energy should be much less than ΔH_u for the over-all process of converting crystalline fibrils to random coil molecules in solution. Under such circumstances, we should be able to apply equation (1) to the transformation without significant error

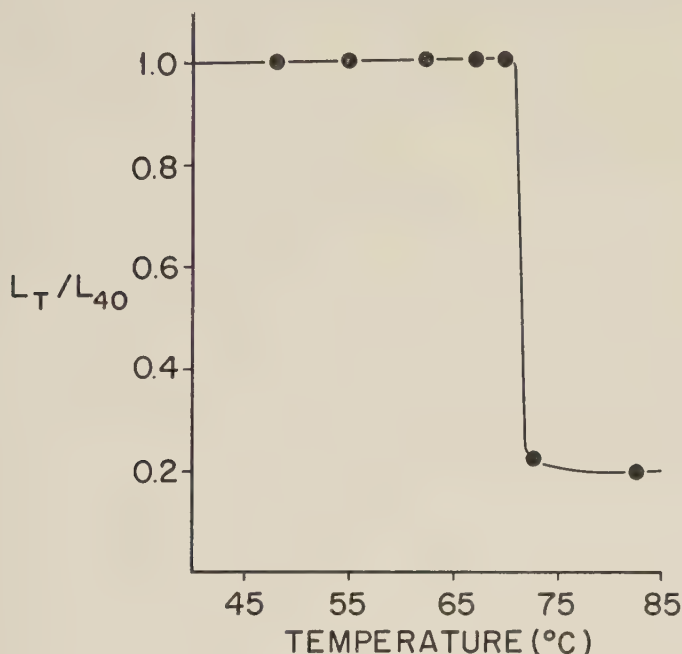


Fig. 3 Length as a function of temperature for formaldehyde-tanned rat tail tendon under a constant small load (E. T. Dumitru, unpublished).

arising from the stepwise nature of the process indicated. This is confirmed by the experimental evidence shown in figure 1.

Consideration of the transformation as a phase transition is particularly helpful in the analysis of the concurrent dimensional changes. Figure 3 shows E. T. Dumitru's results (unpublished) on formaldehyde-tanned rat tail tendon in water. The length was carefully measured as a function of temperature while the collagen supported a constant load,

which in this particular experiment was comparatively small. The sharpness of the shrinkage of collagen, shown in figure 3, is well known. This is a further manifestation of the cooperative character of the phase transition. The influence of stress on the transformation temperature has also been investigated in similar experiments under various loads (Dumitru, unpublished). According to the thermodynamic analysis of such a process in which the phase change is accompanied by a change in length (latent length change), the change in force, f , with absolute temperature, T , should follow the relation

$$\partial(f/T)/\partial(1/T) = \Delta H/\Delta L \quad (2)$$

where ΔH and ΔL are the latent changes in heat and length. An equation equivalent to this one was first derived by Gee ('47). It is an obvious analog of the Clapeyron equation (Flory, '56c). The foregoing results, when treated according to equation (2), lead to a heat of fusion in the neighborhood of 3000 calories per peptide bond, which is in approximate accord with results cited earlier.

It is also to be noted in figure 3 that, prior to shrinkage, the thermal expansion coefficient of collagen is positive and very small. Its value is about that to be expected for a crystalline substance. The narrow interval of shrinkage could be characterized by a very large negative thermal expansion coefficient. Thereafter, when the fiber is completely amorphous, a comparatively small negative thermal expansion coefficient is manifested, in harmony with typical rubber-like behavior. In other fibrous proteins (e.g., elastin or myosin) the shrinkage occurs over a much wider temperature range. The pattern of the thermal expansion coefficient is nevertheless similar to that observed for collagen. Specifically, a small positive thermal expansion is observed at high extensions; at intermediate lengths the coefficient is comparatively large and negative; and at full contraction the coefficient is again comparable to that for a typical rubber. This behavior can be fully explained as a phase transition, which in myosin and

elastin is somewhat broadened by structural variations (Flory, '56b).

The contraction of stimulated muscle may be similarly interpreted as a displacement of the equilibrium between crystalline and amorphous regions. (A qualitatively similar interpretation has been given by Pryor, '50.) X-ray evidence shows the fibers of inactive muscle at rest length to be crystalline, or at least largely so. The degree of crystallinity cannot ordinarily be ascertained from the diffraction pattern alone. The character of the stress-strain curve would suggest that the fibers contain an appreciable, though minor, proportion of amorphous regions. The very low modulus of elasticity of resting muscle indicates, according to the present interpretation, the presence of elements that crystallize on application of stress and others that melt on decreasing the stress.

We suggest that a substance, presumably adenosinetriphosphate (ATP) or one of its decomposition products, is made available by excitation (Pryor, '50) and that this substance tends to complex with certain substituent groups occurring along the myosin chains. The "complex" to which we refer may consist merely of a charged substituent and the associated gegenion, presumed to be large, and necessarily retained in the vicinity of the charged site by electrostatic forces. Accommodation of this complex in the crystalline state is difficult, if not impossible. No such complications should arise in the amorphous state, however. It may be assumed, therefore, that the complex will be formed only in the amorphous polymer. An increase in the concentration of the complexing reagent will consequently shift the crystalline-amorphous equilibrium in the direction of an increase of melting. Contraction must accompany this shift in the melting equilibrium; or, at fixed length, a sharp increase in stress should be observed.

In more quantitative terms, the basic melting relation [eq. (1)] must be modified by appending a term for the change in the chemical potential of the structural unit in the amorphous phase caused by the presence of the complexing reagent.

When this revision is incorporated, equation (1) may be rewritten

$$1/T_m - 1/T_m^\circ = (R/\Delta H_u) (v_u/v_1) (v_1 - \chi_1 v_1^2) + (R N_A/\Delta H_u) \ln(1 + Kc) \quad (3)$$

where N_A is the mole fraction of peptide units bearing the substituent, K is the equilibrium constant for the complexing reaction, and c is the concentration of the complexing reagent.

Available data do not permit a test of this equation. It may be shown, however, that a very marked shift in the equilibrium would result, if K were large, from even a very small *absolute* change in c .

The foregoing explanation of muscle action is an almost obvious extension of the interpretation of dimensional changes in semicrystalline polymeric fibers. A currently popular alternate explanation attributes lengthening of muscle to electrostatic repulsions produced when sites along the polypeptide chains are charged; contraction is thought to occur when these sites are discharged. It would be difficult to account for forces of the magnitude observed, in terms of electrostatic effects occurring in a medium that is about 0.1 *N* in salt. Moreover, electrostatic repulsion should produce *isotropic dilation* rather than unidirectional extension. Also, the electrostatic theory would predict that extension should occur when the substituent groups are charged; whereas, according to the present interpretation, contraction should be triggered by ionization of the substituent groups. The two interpretations lead to opposite predictions, and hence are incompatible.

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PHASE CHANGES AND DIMENSIONAL CHANGES IN FIBRINOGEN AND FIBRIN

JOHN D. FERRY

Department of Chemistry, University of Wisconsin, Madison

FOUR FIGURES

Professor Flory's thermodynamic analysis of the effects of ordering of large molecules and their manifestations in phase changes and dimensional changes is obviously closely related to many phenomena of biological macromolecules. By way of example, I have been tempted to look for applications to something with which I have been concerned for a number of years—the structure and properties of fibrin. This material has some structural features rather different from those of collagen and of insulin, which have been described in most interesting detail in this symposium.

Bovine fibrinogen is generally, although perhaps not universally, believed to be a thin rod about 550 Å long with a molecular weight of 330,000. There is some evidence of a nodular or beaded structure along the rod. When fragments of fibrinogen are split off by the action of thrombin, the surface of the molecule is altered (Lorand, '54). In particular, since negative charges are removed, the electrostatic charge pattern is changed. The principal site of the reaction is believed to be near the middle of one side. This event is followed by spontaneous polymerization, the first stage of which is indicated in figure 1. From physical-chemical measurements we are, I think, fairly certain that the intermediate stage of polymerization is a long rod and that its cross-section area is double that of the initial fibrinogen, as shown. The pattern of overlapping as indicated here has been inferred from various pieces of indirect evidence (Ferry, '54).

Under certain conditions, at high pH or in the presence of appropriate inhibitors, the polymerization stops at this stage. An electron micrograph prepared from such a solution shows rods, straight or nearly so, whose length agrees with deductions from measurements such as flow birefringence, providing visual confirmation of the existence of the intermediate polymers (Kaesberg and Shulman, '53; Siegel *et al.*, '53). Normally, however, the polymerization proceeds further to make bigger fibrils, which of course can also be easily seen in the electron microscope, resulting ultimately in the network structure that constitutes the fibrin clot.



Fig. 1 Suggested arrangement of fibrinogen units in intermediate polymer (Ferry, '54).

The nature of the forces that are responsible for the further aggregation of intermediate polymers has been the subject of only very vague speculation. The feature that differentiates this polymerization from that of insulin, for example, is that the building block of the fibril, instead of being a relatively small molecule, is a rather long rod to begin with. Here is a possible opportunity for applying Professor Flory's analysis, although involving an aspect of it that he did not discuss much in this symposium. The length and inflexibility of a rod, such as the intermediate fibrinogen polymer, will lead to a spontaneous phase separation if there is only a very moderate degree of repulsion between the solute and the solvent, as indicated by a critical value of a certain thermodynamic parameter χ_1 , and it is not necessary to invoke any long-range forces or any specific matching forces to account for the formation of a denser, ordered phase that could become a fibril (Flory, '56).

This might be one important factor influencing the further polymerization of intermediate fibrinogen polymers to produce the strands of the fibrin clot. The question of the form in which the new ordered phase would separate, whether there would be a few big strands or many small ones would presumably

depend on nucleation questions as discussed by Doctor Waugh. Also the question of whether there is any branching of fibers, as is indeed observed in electron micrographs, would depend on such matters.

The result would be (fig. 2) an alignment of the intermediate fibrinogen polymers to form some sort of looser, ordered aggregate. The forces holding the internal surfaces of the fibrinogen molecules together would presumably be fairly specific, involving certain hydrogen bonds, as Professor Scheraga has deduced (Sturtevant *et al.*, '55), but the forces between the intermediate polymers themselves might be far less specific and involve, indeed, primarily just a phase separation.

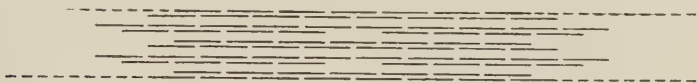


Fig. 2 Suggested arrangement of fibrinogen units, grouped as intermediate polymers, in a fibrin fibril.

There are a couple of things which don't fit in completely with this suggestion. Knowledge of the thermodynamic parameter χ_1 in systems of intermediate polymer shows that, indeed, the value is of such a magnitude that phase separation would be expected, but unfortunately in the presence of certain inhibitors where aggregation does not occur spontaneously, the value of χ_1 is still such as to predict aggregation (Casassa, '56, '57). However, these systems are at best metastable, and a little agitation will induce aggregation in the presence of inhibitors, so perhaps the instability indicated by the thermodynamic analysis is not inconsistent.

The banding structure as shown by Professor Schmitt (this symposium) indicates an accurate lining-up of the individual polymeric units in a fibril. So there must be some degree of specificity leading to proper alignment, even though the forces acting here may represent a rather small proportion of the total free energy change leading to the formation of the aggregate.

I turn now to a quite different phenomenon related to Professor Flory's analysis of dimensional changes. A fibrin clot can be easily compacted from a concentration of the order of 1%, where the polymerization is carried out, to a much denser structure by the expulsion of water. A schematic picture of the compaction is shown in figure 3 (Ferry and Morrison, '47). The thickness of a clot can be enormously decreased to give a fairly dense film of fibrin; here each little string is supposed to represent a fibrin strand that in turn is a bundle of intermediate fibrinogen polymers.

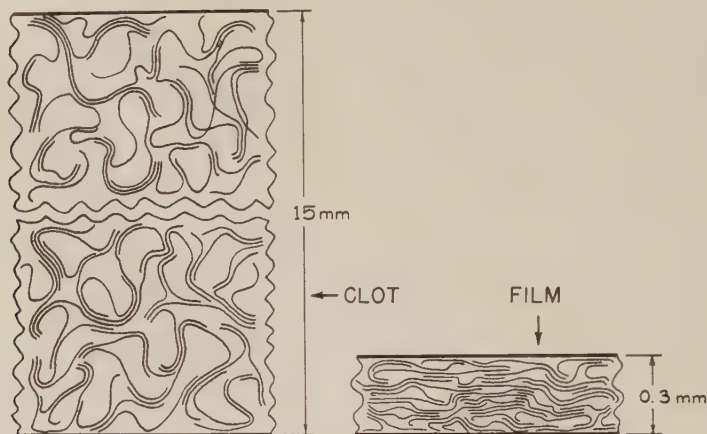


Fig. 3 Schematic picture of compaction of a fibrin clot to form a fibrin film (Ferry and Morrison, '47).

Such a film is quite rubbery and can be stretched to at least twice its initial length. Although the elasticity is not perfect, a large proportion of the deformation can be recovered after the load is removed. When such studies were made 12 or 13 years ago, I supposed that this was rubber-like elasticity, the fibrin strands being engaged in thermal motion and resisting deformation because of the attendant change in configurational entropy. But from what we see now of the detailed structure of fibrin strands, involving units that must be rather stiff, this seems unlikely. More probably, the individual fibrin molecules

are undergoing some intramolecular configurational change, such as from an α to a β configuration. This might still be treated as a phase change being responsible for a dimensional change, except that instead of a liquid-crystalline transformation as discussed by Professor Flory it would be a transition from one crystal form to another.

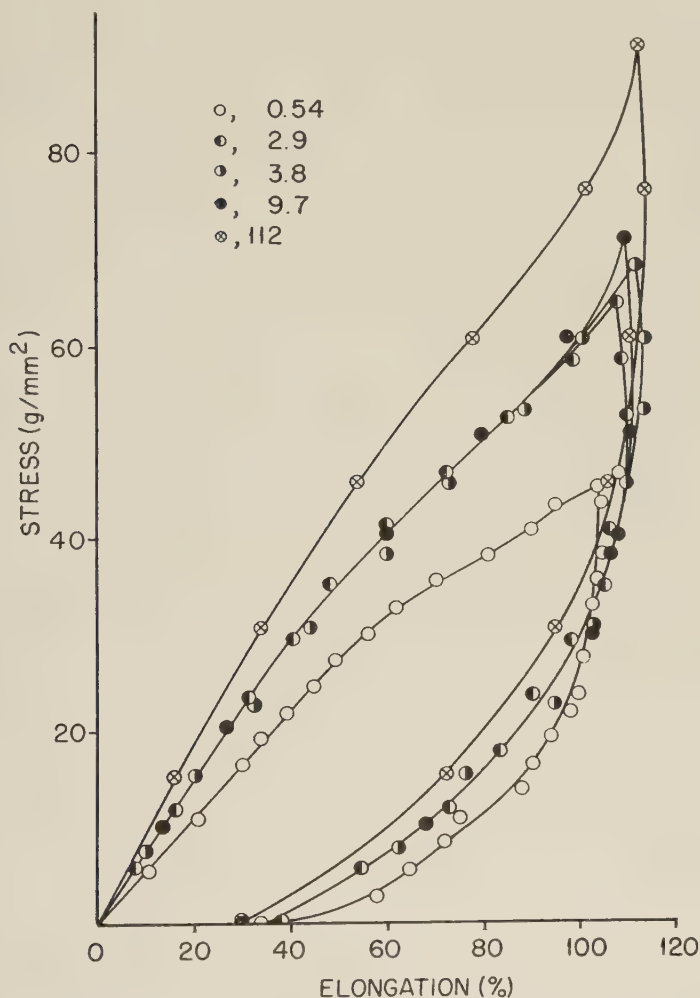


Fig. 4 Stress-strain curves of fibrin film at various rates of loading and unloading (Ferry and Morrison, '47). Loading rate in grams per square millimeter per minute.

Professor Flory has pointed out that in such a case an approach to thermodynamic equilibrium should be very slow, leading to a marked hysteresis in a stress-strain cycle and this, indeed, is what was observed experimentally. Figure 4 (Ferry and Morrison, '47) shows a stress-strain curve in which, depending on the rate of loading, the elongation curve is far removed from the recovery curve, indicating a very slow approach to equilibrium. At a very slow rate of loading and rate of unloading, the hysteresis is somewhat reduced but by varying the loading rate over a factor of 200 it was not possible to approach anything like an equilibrium situation.

These remarks, as you see, raise problems rather than solve them. Their primary purpose has been to call attention to the opportunities offered by the fibrin system for exploring the concepts that Professor Flory has outlined.

GENERAL DISCUSSION

SCHERAGA¹: In our investigation of the possible role of crystallization in the fibrin system from the point of view that Doctor Flory and Doctor Ferry have discussed, we have some preliminary evidence indicating that the phenomenon may differ slightly from that observed during crystallization of synthetic polymers. We are studying the rate of formation of fibrin from fibrin monomer as a function of temperature. In the region of about 30–40°C., the temperature effect is as predicted, but as the temperature drops from 30 to about zero, the temperature dependence of the rate reverses itself. The latter effect is a bit different from that usually observed with synthetic polymers; however, we may still be able to account for the behavior of the fibrin system in terms of a crystallization mechanism.

FERRY: Is this the rate of formation of polymer or the rate of further aggregation of intermediate polymers?

SCHERAGA: This is both steps 2 and 3 combined, without the occurrence of step 1 — we started with fibrin monomer instead of fibrinogen.

¹ H. A. Scheraga, Cornell University.

FERRY: This might be rather difficult to separate into the component parts.

OSTER²: I think there is still further difference between Doctor Ferry and Doctor Waugh; Doctor Ferry is going to have a diffusional controlled process. We have done some rates of clotting as the function of viscosity of the medium by adding sugar, sucrose, or glucose to change the viscosity. The clotting time does not depend on the concentration of these substances *per se*, but is proportional to the viscosity. This would be in line with a diffusional controlled process. It is well known that the clotting time is inversely proportional, or rather it is a second-order reaction. When we tried with Doctor Waugh's case we could not influence the rate. I don't know about nucleation.

FERRY: The rate of polymerization of fibrinogen, of course, is difficult to analyze by measurements of clotting time because of the sequence of processes involved — first the action of the thrombin, then the formation of the intermediate polymer, and finally further aggregation with an eventual gel point.

WAUGH³: We have also been interested in the temperature changes in the fibrinogen-fibrin clotting system and have separated experimentally the stage in which thrombin is activating fibrinogen from the steps of polymerization. The effect of temperature is largely on polymerization. That is, over a wide range of temperature, activation is not markedly changed by temperature, but polymerization is changed. The sensitivity of the polymerization reactions is also indicated by the number of changes in clotting time produced when small amounts of gum acacia or some similar material are added to the fibrinogen solution. In this case, the rate of activation of fibrinogen remains relatively unchanged, although the clotting time may decrease markedly; e.g., the activation rate may change by 10% whereas the clotting time will decrease by perhaps 300%.

FERRY: I would assume that perhaps the formation of intermediate polymer would not be much changed either by the

² G. Oster, Polytechnic Institute of Brooklyn.

³ D. F. Waugh, Massachusetts Institute of Technology.

gum acacia, but it would be the final aggregation step that would be affected.

HAYASHI ⁴: Professor Flory, as I understand it, you have applied your findings from your studies of the crystalline state of proteins to muscle contraction, based primarily on the length-tension diagram of muscle that you showed. If I remember correctly, this diagram is the so-called "passive" length-tension diagram of muscle, and is considered by many authorities to be the length-tension characteristics of passive, noncontractile, parallel elastic components. The "active" length-tension diagram of muscle is quite different, having a peak at L_0 , and supposedly representing the characteristics of the actual contractile components of muscle.

I am wondering, therefore, whether the mechanism you propose should not properly be applied to the passive elements, but not to the actual contractile elements responsible for muscular contraction.

FLORY ⁵: Yes, this is all based on the passive case. It suggests that the primary effect of activation is analogous to that of a change of temperature in the passive case. To be sure, there may be complicating circumstances when muscle is stimulated. I would suggest nevertheless that melting of crystalline order is the basis for contraction in stimulated muscle.

MORALES ⁶: Doctor Hayashi has cited a phenomenon (maximum tetanic tensions at rest length) that I think is not to be explained in terms of the properties of the elementary contractile unit, but rather in terms of the structural organization of units in the fiber. The same can be said of the tension exerted by unexcited muscle. In both instances, I believe there is some interruption of the longitudinal mechanical continuity. These circumstances do not necessarily conflict with Doctor Flory's or any one else's theory of how the elementary contractile units work, but they do warn against the uncritical use of observations on whole fibers in deducing properties.

⁴ T. Hayashi, Columbia University.

⁵ P. J. Flory, Cornell University.

⁶ M. F. Morales, Naval Medical Research Institute.

KIRKWOOD⁷: It seems to me that Professor Flory's ideas on the polyelectrolyte theory of muscle, apart from the details — which may require serious discussion, in no way conflict with this theory. There one is concerned with this symbolic step A plus C and in the ordinary versions of polyelectrolyte theory, one simply supposes that electrical charging or discharging of a macromolecular segment is responsible for going from one stress-strain curve to the other. It has been implied there that one is dealing with a simple random coil cross-linked polymer stress-strain curve. This mechanism could however be grafted onto a more complicated one of the type suggested by Professor Flory in which crystallization might play a role in affecting the stress-strain curve between which one makes the transition.

It is a little hard to see immediately what the effect of the increasing charge would be; there would be two compensating effects. On the one hand, the polyelectrolyte extension caused by increasing the charge would seem to favor crystallization. On the other hand, there would be an opposing effect; i.e., the electrostatic propulsion between the strands might suppress. Then again, if the distance is great enough for a number of ions in between, it might be that the charging up of the chain would favor going to the crystallized state.

FLORY: In my view it should diminish the stability of the crystalline state. Thus factors favoring higher charge should bring about retraction. This is opposite to the conclusion reached by those who seek to resolve the muscle problem in terms of coulombic effects alone. Thus the two interpretations are not mutually compatible, and one cannot be grafted onto the other.

KIRKWOOD: You have reversed your step; i.e., there would be no objection at all to reversing this particular step. Since you have left this very symbolic and undefined, I think that your ideas could be incorporated into the polyelectrolyte theory without drastic implication.

⁷ J. G. Kirkwood, Yale University.

FLORY: I don't like to see it so diluted. Moreover, at about 0.1 ionic strength, such as exists in the environment of muscle, large electrostatic effects are unthinkable. The whole polyelectrolyte hypothesis as applied to muscle seems therefore to be absurd. In reply to the earlier comment by Doctor Morales, the analysis of electrostatic effects in muscle to which he refers is unacceptable in my opinion.

KIRKWOOD: What do you think happens in this step A plus C or its reverse?

FLORY: I think the charging will favor the amorphous (retracted) form, and not the crystalline (extended) form.

KIRKWOOD: Then, as I said, the charging mechanism for this symbolic step can be reversed.

SCHMITT⁸: In striated muscle the two sets of filaments are thought to have widths of about 100–110 Å and 40 or 50 Å. From X-ray evidence, Huxley estimated the center-to-center distance between the large filaments to be about 450 Å. In electron micrographs of thin sections this distance is 200–300 Å, the difference being due presumably to lateral shrinkage during preparation of the specimen.

BEAR⁹: Reliable X-ray measurements of the diameters of normal myosin filaments are not at hand, but a possibly analogous α -protein structure, the "rod" of molluscan paramyosin, has been estimated to be about 100 Å thick (my paper, this symposium). Rough indications are that actin filaments are thinner, 50–100 Å, probably about 60 Å in diameter.

I would like also to make the general comment that evidences of phase changes in muscle have at least been very elusive. Although they cannot be excluded, it has been very difficult to detect them in terms of molecular-chain folding or unfolding by means of X-ray diffraction. Indeed, the only known X-ray signs of change in muscle, related to physiological state, involve equatorial small-angle reflections whose behavior H. E. Huxley interprets as indications of variations in filament or-

⁸ F. O. Schmitt, Massachusetts Institute of Technology.

⁹ R. S. Bear, Massachusetts Institute of Technology.

ganization transverse to the muscle axis without requiring much longitudinal alteration of internal filament structure. These alterations are, however, believed to accompany relative longitudinal displacements of the actin and myosin components.

CARLSON¹⁰: Some years ago I tried to apply the Flory-Huggins-Rehner theory of cross-linked gels to muscle, and undertook the examination of the thermomechanical and swelling properties of muscle. The major difficulty encountered was our failure to achieve an equilibrium. The resting-length-tension curve of muscle changes with time, and a really stable preparation was never obtained. Because of this failure to demonstrate a satisfactory equilibrium it is unlikely that valid interpretations can be made on the basis of equilibrium theories. For this reason, we consider it advisable to investigate the dynamical aspects of muscular contraction in the hope that the data so obtained will be interpretable on the basis of theories of mechanical behavior which are applicable to non-equilibrium states.

FLORY: First on Doctor Bear's well-taken point with regard to the X-ray—it should be understood that the predicted change in the degree of crystallinity will be quite small. There will certainly be less than 10% change of crystallinity over this range of the diagram, and it would be difficult to ascertain such a small change with X rays.

MORALES: In answering Doctor Kirkwood, Doctor Flory has expressed doubt about electrostatic mechanisms on the grounds that they would be ineffective at the ionic strengths thought to obtain in muscle. Doctor Botts and I ('52), and later and in a more refined way Dr. Terrell Hill ('53), have examined this question using independently gathered experimental data, and contrary to Doctor Flory we have concluded that the requisite electrostatic forces could very plausibly arise in the prescribed ionic strength. We would, of course, be very interested in the details of any calculation that supports Doctor Flory's doubts.

¹⁰ F. D. Carlson, Johns Hopkins University.

FLORY: I agree with you but I don't see that it is any objection. You can make the resting muscle correction and make it zero and then go ahead from there. Doctor Hill's analysis has neglected the fact that these fibrils are crystalline. He has treated them as random chains, which is certainly not correct. He has indeed introduced a phase change on one occasion, but a phase change of a very different sort — a liquid-liquid phase change. The similarity to the presently considered transformation is in name only.

DOTY¹¹: I have a purely theoretical question. Has it become a matter of significance in actually plotting the force-tensions curve that the molecules from the end of the crystalline area — from the amorphous area — are oriented, whereas in the ordinary elasticity theory all orientations of the vectors connecting the end are possible? Does that make a significant effect?

FLORY: Yes, it does in certain respects. Suppose a system of highly oriented chains is cross-linked and then the orientation (and crystallinity), for example, is destroyed by melting, and the force measured at a given length. At high degrees of extension the force should become independent of the degree of cross-linkage, which is a little astounding. In other words, cross-linking a highly oriented system should not then affect the force of retraction observed after disorienting.

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OBSERVATIONS ON CONTRACTILE SYSTEMS

J. T. RANDALL

*Wheatstone Laboratory and Medical Research Council,
Biophysics Research Unit, King's College, London*

SEVEN FIGURES

INTRODUCTION

This paper is divided into three main parts. The first part contains extensive physical and biochemical observations on rabbit psoas muscle and is a review of work carried out by Dr. Jean Hanson in the Wheatstone Laboratory and in the Laboratory of Professor F. O. Schmitt in the Massachusetts Institute of Technology, and by Dr. H. E. Huxley in the Cavendish Laboratory, Cambridge, and in Professor Schmitt's Laboratory. The second part of the paper is a brief account of preliminary work on the morphological features of muscle in the chick embryo observed in the electron microscope; this work has been carried out by Dr. S. Fitton Jackson and the writer. The third part of the paper concerns some observations that have been made on fibrous systems in certain protozoa known to be vigorously contractile (Randall, '57).

The paper thus illustrates that the problems of contractility may be studied, not only in adult mammalian skeletal muscle, but also on the developing vertebrate and in the members of the lowest phylum of the animal kingdom. The degree to which modern techniques may be focused on the problem varies enormously in these three fields. In the work described in the first two parts of the paper, for example, it was possible to use the electron microscope, the interference microscope, the method of X-ray diffraction, and also the powerful methods and techniques of biochemistry. In the protozoa, on the other hand, the comparatively small size of the animal renders difficult the precise application of any technique other than

that of the electron microscope. The methods of biochemistry can of course be applied to a few species such as *Tetrahymena* grown in sterile culture, but the results of such experiments generally refer to the metabolism of the whole animal and not to the properties of a particular organelle. Although the results given in the first section of the paper are thus more comprehensive than those of the rest of the paper, this presentation may nevertheless serve to emphasize the wealth of problems of contractility in biological systems that as yet remain almost completely unexplored.

THE STRUCTURE OF A CROSS-STRIATED MYOFIBRIL AND ITS
BEHAVIOR DURING CONTRACTION OR EXTENSION¹

The contractile elements in the fibers of vertebrate skeletal muscles are cross-striated myofibrils that are 1 or 2 μ in diameter. The pattern of the cross-striation is repeated regularly every few microns. The repeat unit is called a "sarcomere," and the length changes in the whole muscle are brought about by length changes in the sarcomeres of its fibrils. This review concerns the fine structure and chemical constitution of the sarcomere of the psoas muscle of the rabbit. Many of the findings have already been published (Hanson, '52; Hanson and Huxley, '53, '55; Huxley, 51, 53a,b; Huxley and Hanson, '54) and have been surveyed in a review (Hanson and Huxley, '55); other results are about to be published (Hanson and Huxley, '57; Huxley and Hanson, '57).

Most of this work has been carried out on glycerol-extracted material. This procedure, originated by Szent-Györgyi, removes many of the water-soluble constituents of the muscle but leaves undamaged the structural proteins of the sarcomere. This remains capable of contracting in a normal manner when it is treated with ATP (Weber and Portzehl, '52), of which a large part is myosin and actin.

¹The work and opinions described here are due to Dr. Jean Hanson and Dr. H. E. Huxley.

Myofibrils consist almost entirely of protein (Perry, '55). Results from Szent-Györgyi's laboratory (Szent-Györgyi *et al.*, '55) show that when myosin is taken out of washed glycerol-extracted fibrils they lose about 68% of their total protein content. Only part of this extracted material (50%) is myosin; there is also another protein fraction (18%) that is called the X protein. The other two main constituents of the sarcomere are actin and the unextractable stroma.

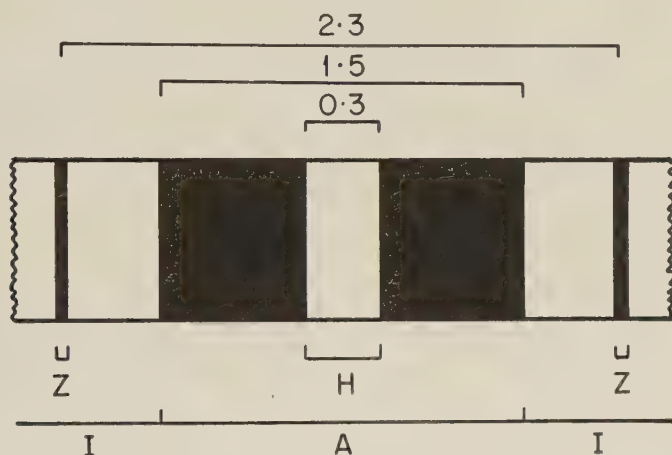


Fig. 1 Diagram of the arrangement of the bands, and their corresponding lengths, in one sarcomere of a myofibril at rest length.

The structure of a sarcomere at rest length will be examined first, and then the location of these four protein constituents within it. Evidence will be produced concerning the precise location of myosin and actin and then, after a description of how the structure of the sarcomere changes when it contracts or is stretched, a new formulation will be given to the old question of how changes in the length of a muscle can be explained in terms of the behavior of its contractile proteins.

In a phase contrast microscope or in a polarizing microscope, the main features of the band pattern can be distinguished. The sarcomere is 2.3μ long and is bounded by two narrow dense Z lines (fig. 1). In the middle is a dense anisotropic

band, the A band, $1.5\ \mu$ long. Alternating along the fibril with the A bands are the I bands, which are isotropic and much less dense. Each I band is bisected by a Z line. In the middle of each A band is an H zone about $0.3\ \mu$ long, which is less dense than the rest of the A band.

Electron micrographs of thin longitudinal sections (Huxley, '53a) show that the A band is characterized by conspicuous longitudinal filaments that do not extend beyond its boundaries. Other material lies between these filaments in most of the A band, but not in the H zone. The filaments in the I band are thinner than those in the A band. In transverse sections (Huxley, '53a) through the H zone, a hexagonal array of filaments is seen. In transverse sections through the A band to either side of the H zone there is a *double* hexagonal array of filaments, one set thick and the other set thin. The thick filaments are continuous with those in the H zone, and each of them is surrounded by six thinner filaments; each thin filament lies in the trigonal position between three thick filaments. In longitudinal sections it is difficult to trace with certainty these thinner filaments along the A band and into the I band, but they are believed to be continuous with the I band filaments (which are of the same thickness) because, after selective extraction of the thicker filaments, there remains an array of thin filaments continuous from the Z line to the border of the H zone (Hanson and Huxley, '53, '55).

The existence of the double hexagonal array of filaments had been deduced from low-angle X-ray diffraction data (Huxley, '51, '53b) before it was seen in electron micrographs (Huxley, '53a). This is important, because the muscle fibers used for the X-ray work had not been subjected to fixation and the other procedures necessary for preparing thin sections; they were, in fact, glycerol-extracted fibers equilibrated with isotonic saline and irradiated while immersed in this solution. The X-ray diffraction data obtained by Huxley show that the distance between the centers of adjacent thick filaments is 455 Å. The diameters of the filaments measured

in electron micrographs (Huxley, '53a) are approximately 110 A for the thick filaments and 40 A for the thin filaments, but their diameters in living muscle are unknown. Figure 2 summarizes diagrammatically the conception of the arrangement of the two types of filaments in the sarcomere derived from all these data.

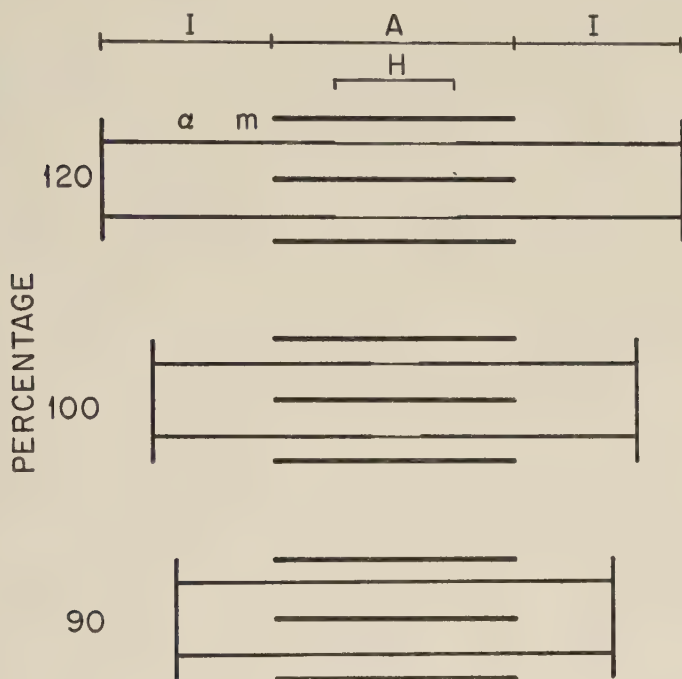


Fig. 2 Diagram of the arrangement of filaments in one sarcomere of a myofibril: top, stretched to 120% rest length while plasticized; middle, at rest length; bottom, contracted to 90% rest length.

The thick filaments are responsible for the high density of the A band. When they have been removed (Hanson and Huxley, '53), there is no longer any distinction between A and I bands, and most of the remaining material is situated in two regions, each extending from the Z line to the border of the H zone. These are the sites of the thin filaments; after this material has been removed (Hanson and Huxley,

'55) the fibril consists of a backbone bearing Z and M lines. This backbone is exposed to sight in the H zone when the A substance is removed.

Thus one recognizes three components of the sarcomere: (1) thick filaments confined to the A band, (2) thinner filaments extending from the Z lines to the borders of the H zone, and (3) the backbone with Z and M lines. It is known that these three components are proteins (Perry, '55), and it is also known that when myosin (together with the X protein) is selectively extracted from the sarcomere the visible result is the disappearance of component (1) without any obvious changes taking place in the other components (Hanson and Huxley, '53). After the A substance has been removed from the fibril it is unable to contract; but if it is then treated with pure myosin, component (2) captures myosin and forms with it a complex that will retract to the Z lines when the fibril is treated with ATP (Hanson and Huxley, '55). This suggests that component (2) may be actin, and, in fact, the procedure used to remove component (2) from the fibril (Hanson and Huxley, '55) is a method (Szent-Györgyi, '51) devised for the extraction of actin from myosin-free muscle. There is also evidence from X-ray diffraction (Huxley, '51, '53b) suggesting that one type of filament may be actin and the other type myosin. In the absence of ATP, i.e., under conditions where one would expect cross-links between actin and myosin, the presence of both components of the double hexagonal array of filaments can be inferred from the X-ray diagrams. On the other hand, living muscle in the relaxed state gives a diffraction pattern indicating that the material of the thin filaments is free to move with respect to the thick filaments; this is what might be expected in relaxed muscle where ATP prevents the formation of actomyosin.

Thus it is considered that the thick filaments of the A band contain the myosin of the sarcomere; the thin filaments extending from the Z lines to the borders of the H zone contain the actin of the sarcomere; and the third component,

left behind when myosin, actin and other soluble materials have been removed, is the stroma.

To establish more firmly this conclusion about the location of myosin in the sarcomere, Hanson and Huxley ('57; Huxley and Hanson, '57) obtained quantitative information by using two complementary methods: (1) interference microscopy of individual sarcomeres and their bands, and (2) analyses by biochemical techniques of the amounts of the different proteins extracted from myofibrils *en masse*.

Interference microscopy is a technique that can give the dry mass of a microscopic object (Davies *et al.*, '54) if its dimensions and the nature of the materials comprising it are known.

$$m = \frac{\phi_w A}{x}; \quad \phi = (\mu_v - \mu_w) t$$

ϕ_w is measured in the interference microscope; A is area and t is thickness; x is 100 times the specific refractive increment. ϕ_w can be measured for the various parts of the sarcomere, and x is known because the fibril is composed of protein. The thickness of the fibril cannot be measured accurately because it is too small. When components are extracted, however, *changes* in mass at particular sites in the sarcomere can be measured; and if the reasonable assumption is made that the diameter of the sarcomere is uniform throughout its length, the mass per unit area in one band can be compared with mass per unit area in another band. Measurements like these are very useful, for if the lengths of the various bands are known, the percentage of total sarcomere protein in each band can be calculated.

Important evidence that myosin lies in the A band is immediately provided when an intact fibril is compared with the same fibril after myosin and the X protein have been extracted from it. Many fibrils have been measured, and in all of them 60-65% of the total material of the sarcomere is removed, 50-55% being the A substance, which is completely extracted, and 10% coming from the "I substance." It will

be recalled that published analyses (Szent-Györgyi *et al.*, '55) by biochemical methods gave the result that the extracted myosin plus X protein make up about 68% of the fibril, 50% being myosin and 18% the X protein. Thus the X protein could not account for more than a small part of the A substance; most of it must be myosin. Biochemical analyses carried out in the Wheatstone Laboratory have given results that are in excellent agreement with those obtained by interference microscopy; when fibrils *en masse* are treated by the same extracting solutions as fibrils under the microscope, an average of 62% of their total protein is taken out (cf. 60–65% by interference microscopy), 51% of it being myosin (cf. 50–55%

TABLE 1
Ratio of mass per unit area in bands

	A : I	H : I	PERCENTAGE DROP IN I BAND
Calculated: Models { 1	1.32	...	61
2	4.37	3.90	0
3	2.87	2.27	31
Observed	2.8	2.2	25

for the A substance measured by interference microscopy), and 11% the X protein.

A second approach to the problem can be made by postulating a number of different ways in which the known components of the sarcomere might be arranged within it; for each postulation, one can calculate the ratios of mass per unit area of the various bands; these calculated ratios can then be compared with actual ratios measured by interference microscopy. Similarly, one can calculate for each model the decrease in the mass of each band on extraction of myosin and the X protein, and then compare the results of these calculations with the results actually obtained by interference microscopy. Table 1 gives the results of some of these calculations and observations.

In model 1, myosin and actin are uniformly distributed throughout the sarcomere and the X protein is the A substance. In model 2, myosin and the X protein are both in the A band, and actin extends from the Z lines to the borders of the H zone. In model 3, myosin is the A substance, and actin and the X protein together extend from Z to H. Clearly the only one of these three models that gives results comparable to those obtained is model 3.

It is well known that actin and myosin *in vitro* can constitute a contractile system, reproducing many of the properties of living muscle. To consider the behavior of actin and myosin *in vivo* in a muscle (where they are located in the sarcomere in the manner shown in figure 2, based on the information summarized), we must know the structural changes taking place in the sarcomere when it contracts or is extended.

Isolated myofibrils contract when they are treated with ATP (Hanson, '52) and the changes taking place in the various bands of the sarcomere while it shortens have been observed under the microscope, photographed, and measured (Huxley and Hanson, '54). It has been found from numerous measurements that the length of the A band does not change over the range 100–65% rest length; shortening is accomplished by retraction of the I bands into the A band until the Z lines are brought to the borders of the A band. The H zone fills up during the early stages of shortening. When the fibril is stretched beyond rest length the length of the A band remains constant, but the I bands elongate and the H zone opens up (Huxley and Hanson, '54). If the A substance is removed from sarcomeres of shortened and extended fibrils, the positions of the other components may be observed (Huxley and Hanson, '54). As the fibril shortens, the material believed to consist of actin filaments moves into the A band, filling up the H zone; as the fibril is extended, this material is drawn out of the A band and the H zone elongates. It should be pointed out that the muscle can be stretched in this manner only when it is plastic (Weber and Portzehl, '52), i.e., under

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conditions where there is reason to believe that actin-myosin links are absent; if muscle in *rigor* or exerting tension is pulled, it resists; it "gives" at the Z lines and the H zone does not open up.

All these observations lead to the conclusion (Hanson and Huxley, '55) that myosin and actin in striated muscle lie in separate filaments (fig. 2) that slide past each other to change the length of the muscle. Contraction is brought about by a mechanism that involves the movement of actin filaments alongside myosin filaments.

MORPHOGENESIS OF MUSCLE IN THE AVIAN EMBRYO²

This part of the paper is concerned with the development of striated muscle fibers investigated by the study of thin sections of avian tissue in the electron microscope. An adequate picture of the fine structure of the sarcomeres at different stages of development is itself of interest, and it is clear that such information may help to resolve the various concepts of the structure of the mature myofibrils.

Preliminary observations were made on developing muscle obtained from the metatarsal rudiments of avian embryos. The material was fixed in the fresh state (Fitton Jackson, '56). It is obvious that electron micrographs of the earliest stages of myogenesis must be interpreted with caution, for it may prove difficult, if not impossible, either to control or know the degree of contraction of the immature muscles on fixation.

In longitudinal sections of myoblasts in a 12-day embryo, the elementary myofibrils consist of three to about fourteen roughly parallel filaments, about 100 Å wide and 300 Å apart. Most myofibrils are bisected at fairly regular intervals of 0.8–1 μ by bands of material 1000 Å wide. Many of the filaments extend through the bands and are often 3–4 μ long; some seem to terminate at or near the bands, but it is possible that such filaments no longer lie in the plane of

² Work carried out by Dr. S. Fitton Jackson and the author.

the section. Presumably the approximately parallel array of filaments that lie between the dense bands may be identified with the A bands of the fully developed muscle fibril, and the narrow dense bands already referred to either may be identical with the Z lines, or may represent contraction bands. In some micrographs, only long filaments are seen, and no bands. This suggests that the individual filaments are formed first and that the dense transverse bands arise later. On the other hand, structures similar to the dense bands and about 0.8μ apart are occasionally present in the sarcoplasm but with no filaments between them.

In the 16-day embryo, the myofibrils so far observed are essentially similar to those just described. At 20 days, however, the I bands and the Z lines of the myofibrils can be distinguished (fig. 3). At this age the dimensions of most of the sarcomeres seen in electron micrographs of longitudinal sections are as follows: the length of the sarcomeres averages 1.65μ , that of the A band 1.18μ , and that of the I band 0.47μ . The A band is composed of one set of filaments, each about 150 A wide, which are spaced about 300–400 A apart; the interfilamentous regions are of medium density. The I bands are of very low density; a few of the filaments from the A bands seem to penetrate the I bands and pass into the dense Z lines, which lie in the center of the I bands. It is not possible to distinguish the same number of filaments in the Z lines as in the A bands, but some filaments are continuous from the A bands through the I bands and the Z lines. No H zone is apparent in the sarcomere.

In longitudinal sections of muscle fibrils in what is evidently a more contracted state, the central region of the A band consists of thicker filaments about 250 A wide and 300 A apart surrounded by less-dense interfibrillar material; the filaments traverse the entire length of the sarcomeres and often penetrate through the probable contraction bands into the adjacent sarcomere.

In transverse sections of the same blocks of muscle tissue, the myofibrils are mainly oval in cross section and are about 0.3 by $1\ \mu$ in size; they are distributed at random in the sarcoplasm (fig. 4). Mitochondria are arranged between the fibrillar columns, but no evidence has been obtained to support the view that mitochondria give rise to the myofibrils. Each myofibril in transverse section consists of one clear, well-defined set of filaments in approximately hexagonal array (fig. 5) and ill-defined material occupying irregular interstitial positions. The filaments are 150 – $250\ \text{\AA}$ in diameter and are about $300\ \text{\AA}$ apart; these measurements are in agreement with the fine structure seen in longitudinal section. It is not yet clear, however, whether the ill-defined material is related to the interstitial bridges proposed by Hodge ('55) and which are believed to be laterally joined to the filaments, or whether it is identical with the thinner filaments lying in trigonal positions relative to the thicker filaments, as suggested by Hanson and Huxley ('55) in adult rabbit muscle. We hope that further investigation will clarify this point.

Where the fibrils are adjacent to the cell surface, the Z lines sometimes appear to be connected with the surface membrane, a structural feature that may be important in the transmission of electrical impulses at the neuromuscular junction.

In preliminary light microscope measurements of similar fibrils from 20-day embryos at rest length, the average length of the sarcomere is $2.1\ \mu$; the A band is $1.35\ \mu$, and the I band $0.75\ \mu$. Thus it would appear that the observations on the 20-day embryonic muscle in section must apply to fibrils at about 80% rest length if allowance is made for a proportional shrinkage during preparative treatments. This view is supported by the fact that the I band is less than half the length of the A band, since Spiro ('56) has shown that in chick muscle fibrils the I band should not be less than half the length of the A band when the myofibrils are at rest length. The degree of contraction in material of this age may account

for our failure to observe an H zone, which is usually not apparent in fibrils contracted to less than 90% of rest length.

If it is assumed that the length of the sarcomere at rest length remains constant throughout all stages of development, the observations on the 12-day embryos must refer mainly to contracted material at 65% or less of rest length; the dense bands would therefore correspond to contraction bands. It must be emphasized, however, that it is not yet clear whether the sarcomere increases in length during development.

The present work indicates that at first the main constituents of the myofibrils are filaments, 100–150 Å wide, that form the backbone of the myofibrils, and that may extend considerably further than the length of the mature sarcomere. After further differentiation of the tissue, the arrangement of the bands within one sarcomere is essentially similar to that of vertebrate muscle with the possible exception of the presence of the H zone, but the final array of the component filaments is incomplete.

CONTRACTILE SYSTEMS IN PROTOZOA

A number of the ciliate protozoa have long been thought to possess structures responsible for their contractility. For example, the most elementary observations of living *Spirostoma* show that the animal must possess some means of extremely rapid contraction. The structures are known as myonemes and have been inferred in the past from the light microscope study of living and of fixed-and-stained material. The electron microscope observations that have now been made on thin sections of appropriate material (Randall, '56, '57) show that the width of the myonemata is close to the limit of optical resolution, which no doubt accounts for the conflicting accounts that have appeared in the literature.

A study of the fine structure of *Spirostomum* and *Stentor* is in progress (Randall, '56, '57) but here it is appropriate to refer to the only structures that, by their fibrous or sheet-like character, seem likely to exercise a contractile function in these primitive animals, for it is necessary to point out

that electron micrographs do not in themselves provide evidence of contractility. The comparatively small size of the protozoa makes it difficult, if not impossible, to extract particular structures such as the myonemes for more precise examination in the way, for example, of Hanson and Huxley's experiments on rabbit psoas muscle.

If a transverse thin section is made of *Spirostomum* so as to include part of the ectoplasm of the periplast, it is found that the external shape follows the ridges and furrows so characteristic of the living animal. Distinctive structures are arranged asymmetrically on one side of each ridge. Longitudinal sections show that these structures are extremely long and probably extend the whole length of the animal; they are identified by their characteristics and position with the myonemes described by older workers. Each myoneme appears to consist of a number of fibrillar ribbons or sheets, often about ten. One side of each ribbon is attached to the plasma membrane. The observed material is invariably in a contracted state; the width of the ribbons when flattened out is about 5000 Å. The chief feature of each ribbon is its fibrillar nature, the fibrils being about 250 Å in diameter.

The contractile structures of *Stentor polymorphus* (fig. 6) are not unlike those already described for *Spirostomum*; they also are situated asymmetrically in each ridge and are formed of systems of closely associated narrow sheets or ribbons. The ribbons are uniform in cross section and do not show the coarse fibrillar structure evident in *Spirostomum*. Comparison of micrographs suggests that each myoneme is composed of 20–30 sheets about 130 Å thick and 5000 Å wide.

The fine structure of *Carchesium* is also being investigated (Randall, '56); it is a peritrich and each branch of the stalk is independently contractile. The stalk consists of two distinct parts: an annulus threaded by numerous longitudinal tubular filaments marked with a periodic striation and a central core or canal that appears to be continuous with the endoplasm of the zooid and is separated from the annulus

by a single membrane. The annular filaments have a transverse striation of somewhat irregular spacing, the value of which generally lies between the limits of 300 Å and 700 Å (fig. 7).

It has generally been considered that the central core of the stalk is the seat of the contractile mechanism, for the contractile stalks of *Carchesium*, *Zoothamnium*, and *Vorticella* each have a canal, whereas it is missing in the noncontractile stalks of *Opercularia* and *Epistylis*. Although a careful search has been made in *Carchesium* for the existence of possible contractile fibrils in the central canal of the stalk, no convincing evidence of their existence has yet been found. Thus in the present state of knowledge it is possible only to speculate about the nature of the contractile mechanism. The striated filaments observed in the annulus of all the genera mentioned (Randall, '56; Fauré-Fremiet and Rouiller, '55) may be potentially contractile. Contraction may, however, be achieved only through the action of a specific substance diffusing outward from the canal. Alternatively, but less likely, the filaments may not themselves be contractile but may perform a strengthening or supporting function. Contractility might then be achieved through an as yet unverified system of fibrils within the central canal.

GENERAL DISCUSSION

MORALES³: My colleagues and I (Morales *et al.*, '55; Morales and Botts, '56) have cited a number of reasons for doubting the model originally proposed by Huxley and Hanson. The most primitive of these is the shortening of the threads drawn from extracted contractile protein under the influence of ATP although they lack the interlacing myosin array-actin array postulated by Huxley and Hanson. I wonder whether Dr. Randall agrees that this circumstance is very embarrassing to the theory?

RANDALL: The model proposed for striated muscle has two main features: (1) myosin and actin are in separate filaments

³ M. F. Morales, Naval Medical Research Institute.

and (2) these filaments are arranged so as to produce an alternation of A and I bands along the length of the muscles. This second feature is peculiar to striated muscle. As Hanson and Huxley have pointed out (Hanson and Huxley, '55, p. 238), the absence of A and I bands in smooth muscles or in the threads made from extracted protein, does not preclude the possibility that in these contractile structures, as in striated muscle, myosin and actin are located in separate filaments.

KIRKWOOD⁴: Dr. Morales mentioned that actin was supposed to interpenetrate the myosin. Is there any evidence at all, other than pure phantasy, that the actin filaments do not go continuously from Z to Z?

RANDALL: Well, the optical evidence for the variation of the density along the bands that I gave, together with the evidence deduced from the work with the interference and electron microscopes, supports this contention.

KIRKWOOD: Can you really tell that the actin filaments are interrupted? There was no evidence from your mass density.

RANDALL: A transverse section through the H zone is quite different from a section through the neighboring region, as seen in the electron microscope.

ALBERT SZENT-GYÖRGYI⁵: I have seen the preparations of Huxley. They are very beautiful but it is one thing to see something and another to make a model. It becomes increasingly difficult to make a model. The more we know the more difficult it becomes because a model has to take account of all data, and this model absolutely contradicts basic physiological observations. That is the reason Andrew Szent-Györgyi, D. Mazia, and I asked whether there is no other unknown protein in muscle that could account for the observations of Hanson and Huxley. A new "X protein" came out in big quantities. Since then we have been struggling with this X protein; it is a very difficult one.

⁴ John G. Kirkwood, Yale University.

⁵ Albert Szent-Györgyi, Marine Biological Laboratory, Woods Hole.

RANDALL: The X protein is not present on their evidence in the A band in anything like a sufficient amount to account for the interference microscope measurements. If it were it would be a different matter.

SZENT-GYÖRGYI: This new protein has very unpleasant properties, which makes the estimation of how much there is very uncertain, and so it is too early to draw any conclusions from density measurements on distributions.

RANDALL: We have been very careful to follow all your methods on this extraction and so on, as you know very well, I think. I am no person to dispute what you say, but I know my colleagues have been very careful about this sort of thing.

SZENT-GYÖRGYI: We ourselves distrust the accuracy of our own figures.

RANDALL: What do you think about the importance of studying the embryo tissue in this connection? It would seem that there one can follow the development of the muscle in stages that might throw interesting light on all these conceptions.

SZENT-GYÖRGYI: Study of embryonic muscle seems exceedingly interesting, but here too you have the general difficulty that when you see something in the electron microscope, you do not know what it means, and it is exceedingly difficult to interpret pictures. Embryonic tissues tell us that there can be contraction without A bands, that *first* the muscle contracts and *then* it begins to form an A band. So the A band is not essential for contraction. This X protein is globular but the globules very easily join to filaments. My guess is that this X protein is dispersed at the time when the muscle begins to contract and is hammered into a sheet, into the A band by contraction. I don't say that this is necessarily correct. I say that this is an alternate picture, and the trouble is we can make too many alternate pictures without being able to decide which is the correct one.

SCHMITT⁶: Dr. David Spiro, in our laboratory, has deduced from his electron microscope studies of thin sections

⁶F. O. Schmitt, Massachusetts Institute of Technology.

of striated muscle, an arrangement that would be in agreement with model 3 as shown by Professor Randall. It is very difficult to make out very clearly the structure in the I band but it seems probable that the poorly resolved, very thin (~ 50 A) filaments are composed primarily of actin.

In interpreting the significance of the structure seen in the A band, Spiro has stressed the importance of maintaining rest length in the fibers at the time of fixation; he believes that most published electron micrographs of muscle were obtained from partially contracted myofibrils. In rest- or equilibrium-length fibrils, Spiro finds relatively thick filaments in the region of the H bands, in agreement with most authors. However, in the remainder of the A band, he finds about twice as many thin as thick filaments. As shortening proceeds, the thick filaments grow in length toward the I band at the expense of thin filaments until only thick filaments are seen. It is believed that several of the thin filaments aggregate, along with myosin macromolecules, to form each thick filament.

Spiro's interpretation is that the thin filaments, presumably composed primarily of actin, course continuously between H bands of adjacent sarcomeres, through the I bands. In resting muscle, the myosin occurs primarily as long, thin macromolecules (23×2300 A, according to Weber) oriented essentially parallel in the A band. In contraction, the actin filaments and the myosin macromolecules interact strongly, possibly by forming a tightly wound coil. This would draw the thin filaments into the A band, causing a lengthening of the thick filaments (of actomyosin) and a reduction of the number and length of the thin filaments.

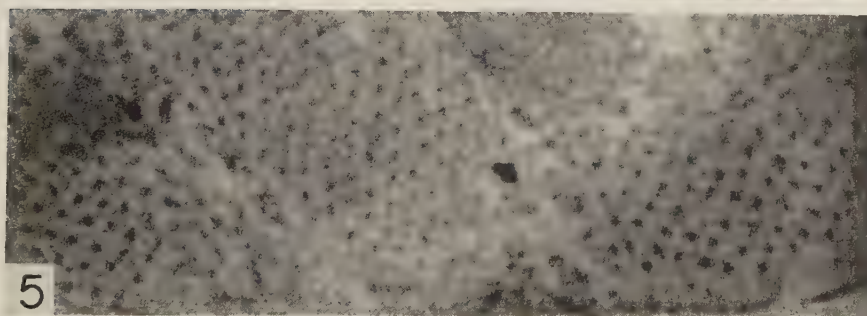
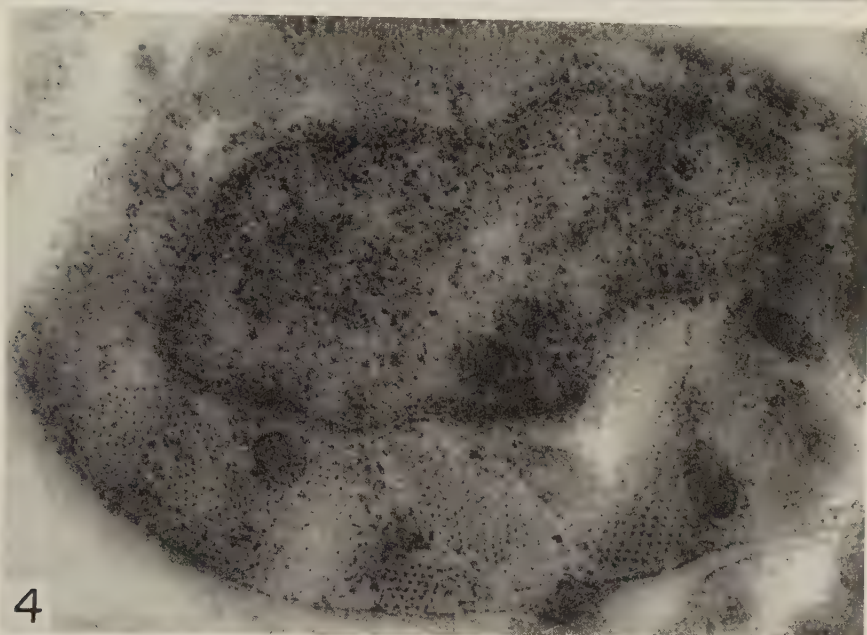
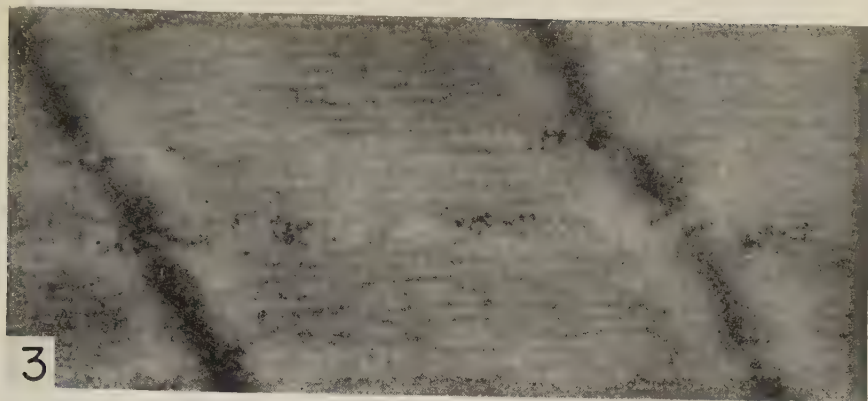
Spiro's electron micrographs of partially or fully contracted myofibrils are similar to those of Hanson and Huxley (and also of Hodge). However, from his study of uncontracted myofibrils, he concludes that the interaction of the myosin or myosin-rich fibrous component with the actin or actin-rich thin filaments is caused by a supercoiling that in turn must reflect some kind of complementarity of molecular structure.

By occupying certain sites on the fibrous molecules, ATP prevents this close association between actin and myosin, the tight coils unravel and the sarcomere elongates. This interpretation substitutes close interaction of fibrous macromolecules due to structural complementarity for the long-range type of interaction proposed by Hanson and Huxley to explain contraction on the basis of interdigitating filaments of actin and myosin.

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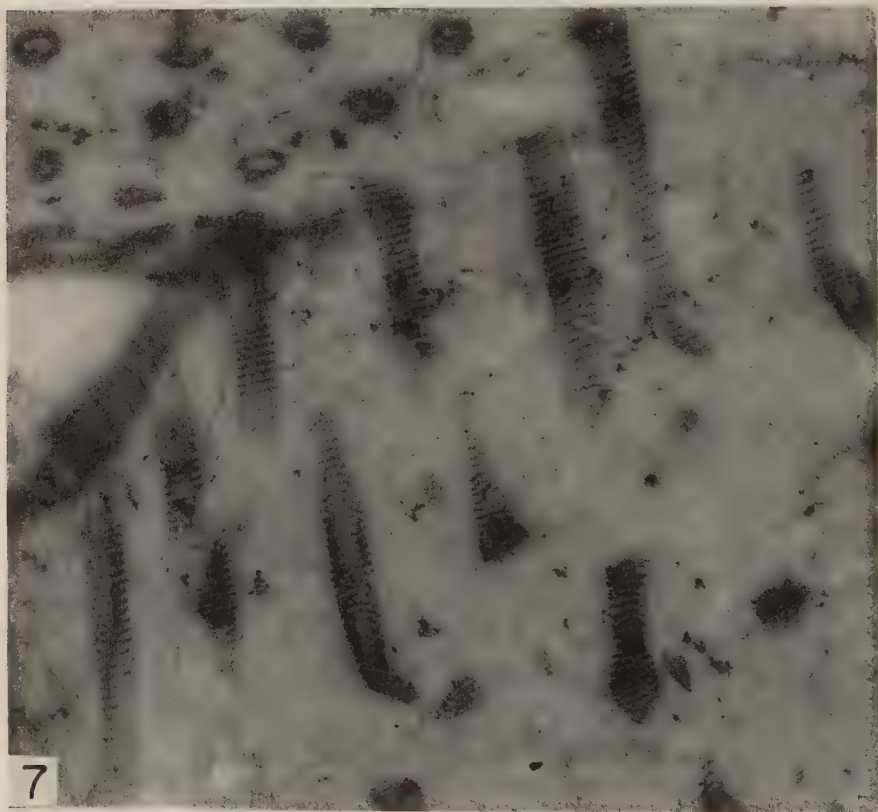
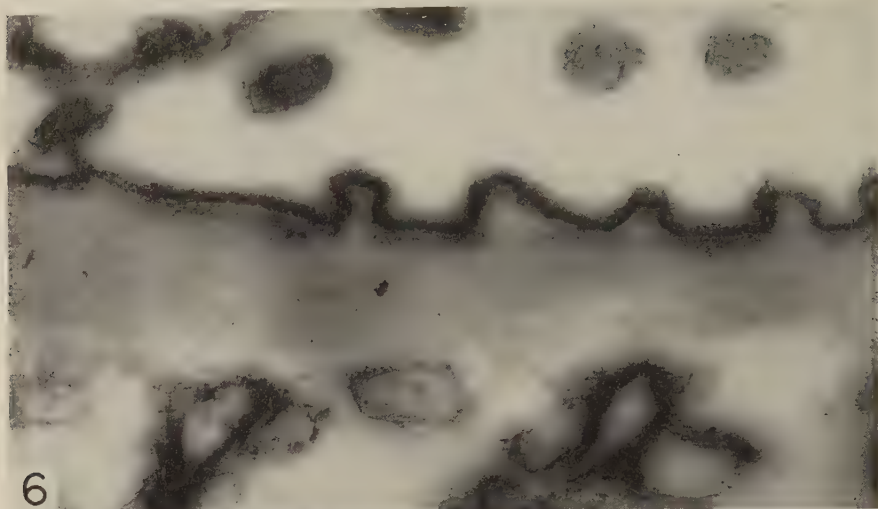


EXPLANATION OF FIGURES

3 Electron micrograph of a longitudinal section of a myofibril from a 20-day embryo. The I bands and Z lines are apparent and the A band is composed of one set of filaments, about 150 Å wide and 300-400 Å apart. ($\times 42,000$)

4 Electron micrograph of a myoblast in a 20-day embryo showing a centrally situated nucleus and many myofibrils, cut in transverse section, distributed in the sarcoplasm. ($\times 20,000$)

5 Enlargement of part of figure 4, which shows the arrangement of the filaments and the interstitial material of the myofibrils. ($\times 80,000$)



EXPLANATION OF FIGURES

6 Electron micrograph of a section through the peripheral zone of *Stentor polymorphus*. Numerous filaments are lying just beneath the pellicle and they probably represent sheets or ribbons in longitudinal section. ($\times 30,000$)

7 Electron micrograph of a section through the annulus of the stalk of *Caracusium*. The tubular filaments, marked with periodic striations, are well defined. ($\times 40,000$)

LABILE COLLOIDAL COMPLEXES OF THE CYTOPLASM

NORMAN G. ANDERSON

Biology Division, Oak Ridge National Laboratory,¹ Oak Ridge, Tennessee

EIGHT FIGURES

INTRODUCTION

The living cell is a system of colloids, bathed in an aqueous medium that contains a small concentration of inorganic ions and of relatively low-molecular-weight organic substances, most of which bear a negative charge. The distribution of molecular weights is bimodal, with very few substances whose molecular weights lie between the coenzymes, complex lipids, and nucleotides; and proteins and nucleic acids. If most reactions proceed through the formation of complexes (Johnson *et al.*, '54), then consideration of cellular reactions and processes in terms of complex formations seems logical. These reactions fall into two general types; the first, between macromolecules and substances of fairly low molecular weight includes most enzymic reactions, specific binding of ions or dyes. The second is limited to interactions of two or more large molecules, including a few enzymic reactions and interactions, especially between protein molecules, that range all the way from certain exquisitely specific serological reactions to the interactions generally occurring in concentrated protein solutions. In this paper the second class will be discussed almost exclusively.

INTERACTIONS OF LARGE MOLECULES

If we are to discuss the general topic of the relation of colloidal complexes to cell physiology, we must know what prob-

¹Operated by Union Carbide Nuclear Company for the U.S. Atomic Energy Commission.

lems on the cellular level are involved. One of the oldest and most fundamental questions of cellular growth and organization is whether each macromolecule is duplicated *in situ* or whether such production is the function of some cellular organelle (Conklin, '40). Superficially, the first possibility is far the simpler. The second is however supported by the findings that (1) cellular proteins turn over at different rates, (2) excised cytoplasmic organelles can be regenerated, and (3) incorporation of amino acids into proteins appears to be largely confined to the microsomes (Hultin, '50a, b; Siekevitz, '52). The segregation of synthesis into discrete centers raises the problems of (1) how the synthesized molecule is detached from the synthetic site or template if such there be, (2) if it is to form structures with other proteins, how it is kept in solution until the proper site is reached, (3) how a protein or other macromolecule is held in place in a cell structure, (4) by what method information can be "fed back" from stable structures to synthetic sites to adjust the level of production, and (5) how the activities of large molecules can be manipulated to affect and control either their individual or their combined activities.

The theme of this discussion is that a key to many of these problems and several to be discussed may be found in the ability of many cellular colloids to form complexes whose bondings range from extreme lability to such firmness that partial degradation occurs before dissociation. In papers by Oster, Overbeek, and Kirkwood (this symposium), a number of mechanisms and forces have been discussed that can bind macromolecules together with varying degrees of tightness. Of special interest is the precipitation of globulins through interaction of oppositely charged areas on the same molecule, as mentioned by Overbeek. This mechanism is sensitive to salts, changes in dielectric constant, and temperature. The charge-fluctuation theory of Kirkwood and Shumaker ('52) provides additional forces. Other contributors to this symposium have demonstrated and discussed the interactions of

discrete molecules that give rise to long fibrils, the ordering of fibrils to produce various structures, and the role of fibrils in several very difficult morphological problems. Unfortunately, collagen, cellulose, and most of the proteins studied in model systems, with the possible exception of those derived from muscle, cannot be considered as being characteristic constituents of cytoplasm.

Little attention has been given, however, to the importance of more-labile complexes, especially the soluble ones, or to the existence of wide ranges of intermolecular specificities and binding forces. I propose to review the role that these are believed to play in those processes and properties characteristic of that ultralabile system — the living cell. For the reactivity, responsiveness, and variability of the cytoplasm can find its explanation only in a sensitive series of reactions such as may be mediated through very labile complexes.

The aim of the cell physiologist is to describe and explain the complex and various properties, activities, and responses of living cells on a molecular level. This may be attempted in two ways. First, the properties of relatively pure substances isolated from cells may be studied in the hope of reconstructing cellular processes in terms of such properties. Although numerous tissue enzymes have been isolated and characterized, there is no clear picture of cellular molecular populations, the groups into which they fall, and the ranges of molecular weights, charge densities, shapes, and compositions. Since the isolation of a particular cellular protein has generally been guided by an enzymic activity, a radioactive label, or some distinguishing group, it is evident that many tissue proteins that are either not enzymes, or whose activities have not been discovered, have been overlooked. Furthermore, the proteins that have been isolated cannot as yet be studied with sufficient detail. Although the synthetic approach has been useful, for example, in attempts to explain certain physical properties of nuclei and chromosomes in terms of deoxyribonucleic acid (DNA) molecules (Anderson, '56a) and in the study of the re-

lation of muscular contraction to muscle proteins, too few cellular proteins have been studied. The data that can be obtained at present are neither precise nor detailed enough to allow cytoplasmic processes to be reconstructed from the properties of pure substances. The second approach is an analytical one by which we attempt to isolate cellular fractions that still retain some of their intracellular properties and to extract from such fractions the substances responsible for those properties. Only this second approach now appears generally feasible; the results of certain studies performed to explore it will be discussed.

At this point, a brief discussion of the specific types of problems that we hope to approach seems in order: (1) sol-gel changes in the cytoplasm, (2) responses to heat and cold, (3) certain effects of alterations in pH , (4) the effects of anesthetics, (5) mechanisms for controlling reaction rates, and (6) mechanisms for forming structure. If isolated systems are to be devised for studying these problems then the choice of a preparative method assumes great importance.

The reactions of juices prepared from yeast and muscle have long been studied and related to intracellular events (Bernard, 1875; Warburg and Wiesel, '12; Winterstein, '26). The tendency has been, however, to discount the results of such studies on the grounds that "protoplasm" could not be isolated and that preparations studied represented the autolytic degradation products of "injured" cells. The concept that the cytoplasm could be studied meaningfully only inside the cell necessitated an ultramicroanalytical approach involving micrurgical and histochemical methods and refined optical and chemical techniques. The localization of a substance is one thing, however, and its characterization quite another. Unfortunately, sufficient material for characterization can be obtained only from large masses of cells. What is required is that contemporary methods of cell fractionation be adapted to the problems listed. But there are questions of whether meaningful results can be obtained with such methods and whether

the cytoplasm is too labile to allow its properties to be reflected in isolated preparations. During a considerable period of the history of cell physiology the results obtained by methods now in use would probably not have been accepted as relevant to cellular processes. Their acceptance today was brought about largely by the extensive biochemical work that has already been done with these methods.

There is no escape from the rapid autolytic changes that occur when cells are ruptured, but low temperatures and rapid handling minimize them. The choice of a suitable medium is a complex problem that is still far from solved. If the properties of interest in cytoplasmic colloids are not so capricious that they disappear completely the instant the cell is broken, then some of the intracellular properties must be retained in the brei. If cell fragments are partially "dead" they must also be partially "alive." If properties associated with vital activity can exist in degrees, some remnant of them can be studied *in vitro*. The difficulty is partly conceptual.

The next step is an examination of materials obtained from broken cells for evidence of those intracellular reactions of interest here. If these can be demonstrated *in vitro*, the task of elucidating their molecular basis may be started.

EXPERIMENTS WITH CELL FRACTIONS

For a number of reasons work with whole-tissue breis is difficult. We have therefore attempted to characterize a soluble fraction from perfused rat liver.

The soluble fraction or phase was prepared by a method (Anderson, '56b) in which the liver is very rapidly perfused, fragmented in a shearing field, and centrifuged so that the nuclei and nearly all the mitochondria are sedimented within 13-14 minutes after the death of the animal. The supernatant fraction was then centrifuged for 1 hour at 40,000 rpm ($105,000 \times g$ at the center of the tubes, $R_{\min} = 3.8$; $R_{\max} = 8.1$ cm) and the upper lipid layers discarded. Eighty-eight per cent of the nitrogen of the final supernatant was nondialyzable.

The color was clear yellow with absorption maxima at 268 and 407 $m\mu$ before dialysis and 278 and 407 $m\mu$ after dialysis. A greater part of the 260 $m\mu$ -absorbing material is dialyzable.

The marked temperature sensitivity of many animals is not easily explainable in terms of the denaturation of proteins, since heat death has been observed as low as 11.5°C. in the cold water crustacean, *Pandalus borealis* (Abercrombie and Johnson, '41) and as high as 50–80°C. in hot water algae (Heil-

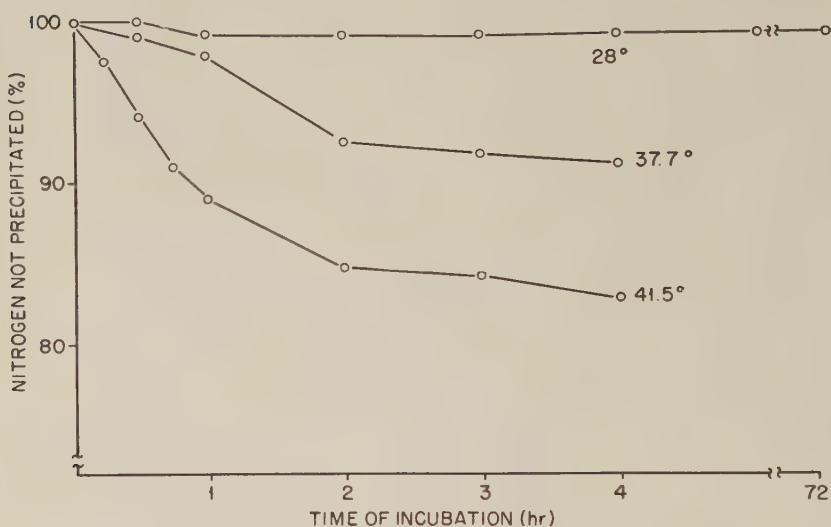


Fig. 1 Effect of incubation at times and temperatures indicated on rat liver soluble proteins (ratlivsol). Nitrogen is expressed as the percentage of the original nitrogen remaining in the supernatant fluid after centrifugation of the incubated preparation.

brunn, '56). Temperature also markedly affects the viscosity of many cells, usually producing an increase at both high and low temperatures (Heilbrunn, '56). The effect of incubation of rat liver soluble proteins (ratlivsol) at several different temperatures on the amount of nitrogen precipitated is shown in figure 1 (T. Makinodan and N. G. Anderson, unpublished). At 41°C., about 2% of the nitrogen precipitates in 15 minutes. At 4°C., 8–9 days are required for the same amount of precipi-

tation. The rate of precipitation increases rapidly as the thermal death temperature ($40^{\circ}\text{C}.$) (Heilbrunn, '52) for the rat is approached. Owing to the high temperature coefficient, these results probably cannot be explained simply as the results of enzymic digestion of liver proteins. Although enzymic action may be involved, some change more nearly related to melting probably occurs. This is in keeping with the suggestion that temperature tolerance is related to the melting point of body fats (Heilbrunn, '24) or some other physical change. Electrophoretic studies on soluble liver protein preparations before

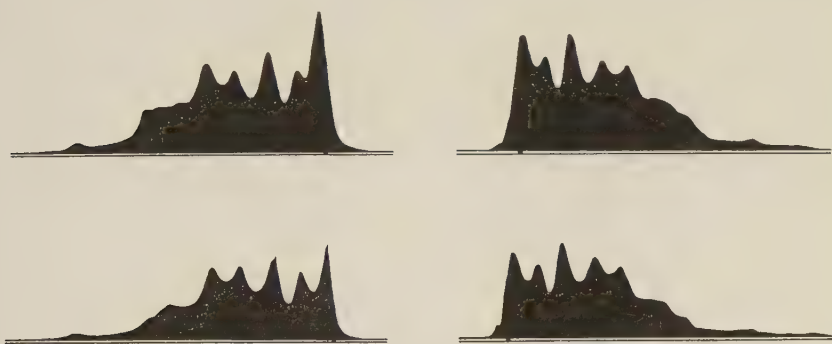


Fig. 2 Effect of incubation for 2 hours at $37.7^{\circ}\text{C}.$ on electrophoretic patterns of ratlivosol. Upper patterns, control; lower patterns, experimental. Ascending limbs on left side. $\text{pH } 7.5$; $\mu = 0.1$.

incubation and after incubation for 2 hours at $37.7^{\circ}\text{C}.$ does not reveal that any component is uniquely involved in the formation of a precipitate (fig. 2), although one of the leading components does show a considerable decrease. The marked sensitivity of the ratlivosol preparation to heat with a rapid increase in the rate of precipitate formation near the temperatures causing heat death in the intact animal suggests that useful information on the cause of heat death may be obtained from isolated preparations. As will be discussed, the changes observed are believed to be caused by disaggregation of soluble complexes.

The results obtained with heat suggested that further exploratory experiments should be undertaken. When dialyzed ratlivosol was frozen and thawed, a marked precipitation was observed. From studies on the factors that prevent the formation of precipitates after freezing and thawing, we concluded that precisely the same substances — sugars and glycerol — that are well known as protectants for freezing whole cells protect the ratlivosol preparation. When ratlivosol is frozen with increasing concentrations of salt, the amount of precipitation is proportionately decreased. This may explain the higher survival of tumor cells when they are frozen slowly. Since water crystallizes first in the intercellular spaces, the cells are suspended in slowly increasing concentrations of salts.

A peculiar type of gelation is observed in a dialyzed ratlivosol preparation frozen and thawed four times and then examined electrophoretically (fig. 3 D). Undialyzed ratlivosol, which contains sucrose, was not appreciably altered by this treatment. The control sample from the same ratlivosol preparation was kept at 4°C. and is shown after electrophoresis under identical conditions. Again the ratlivosol system is like the cell in that it is sensitive to freezing. Some precipitation is occasionally observed before freezing actually begins.

Next, to explore further the parallels between the cell and ratlivosol, we examined the effect of changes in *pH*. In a variety of cells, a slight lowering of *pH* increases the viscosity of the cytoplasm (Barth, '29; Lewis, '23; Heilbrunn, '56). Samples of ratlivosol were mixed with buffers of different composition and *pH* (fig. 4), centrifuged for 30 minutes, and the amount of nitrogen per milliliter of supernatant fluid determined. The results indicate that a large fraction of the ratlivosol nitrogen is insoluble at around *pH* 5 and is more soluble on either side of this *pH*. It appears likely, therefore, that the increase in cell viscosity observed when the cells are subjected to slightly acid solutions is caused by the decrease in solubility of the fraction precipitated in these experiments. The major portion

of the material precipitated is from one peak, as shown in figure 5. This is the same peak that lost material during incubation.

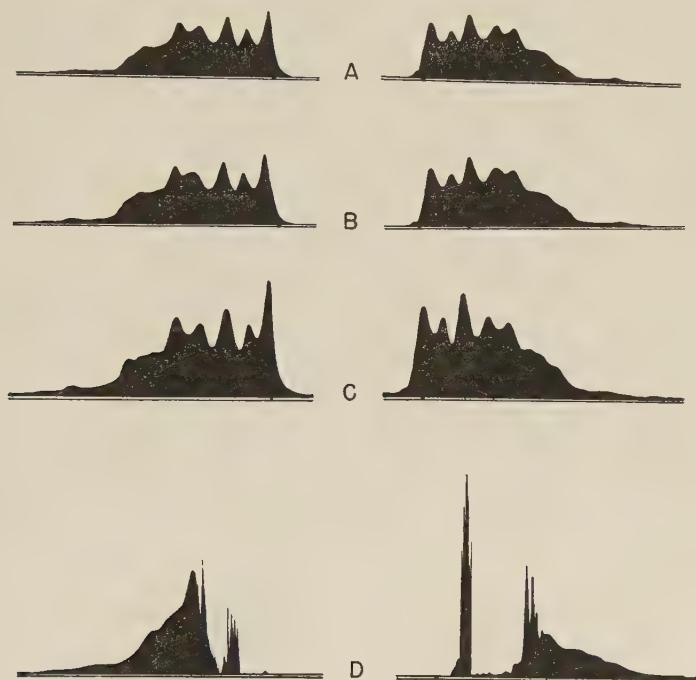


Fig. 3 Effect of freezing and thawing on dialyzed and nondialyzed ratlivosol. A, control; B, same preparation frozen and thawed four times before dialysis; C, control preparation; D, same preparation frozen and thawed four times after dialysis against electrophoresis buffer, $\mu = 0.1$. A large part of the difference observed between the dialyzed and nondialyzed preparations is believed to be caused by the presence of sucrose in the latter. The spikes in the D patterns are caused by formation of a very weak gel whose sharply defined edges could be observed after the electrophoresis cell was removed from the low-temperature bath.

Cells are surprisingly sensitive to electric currents. During an early series of studies on the electrophoretic properties of ratlivosol, we observed that the pattern disappeared in a space along the descending limb just below the starting boundary, as shown in figure 6 (veronal buffer, pH 8.6), and a white precipitate formed in this space. The width of the precipitate

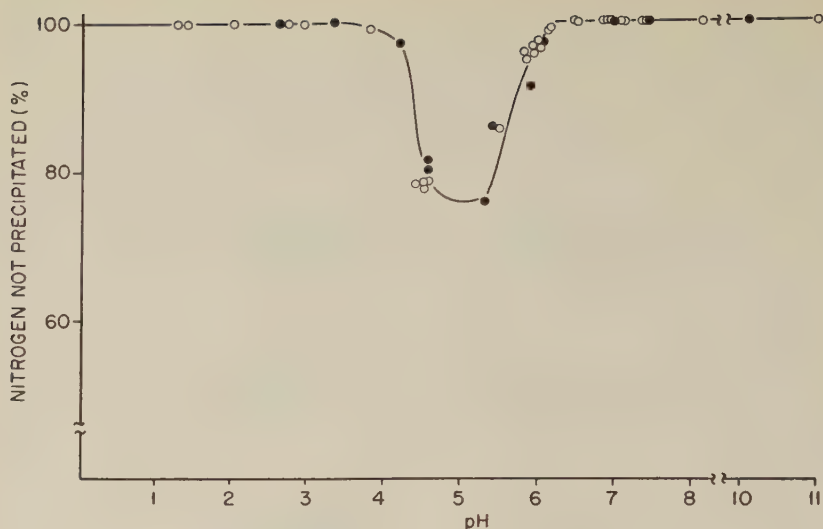


Fig. 4 Effect of pH on solubility of ratlivsol proteins. Ratlivsol was mixed with equal volumes of phosphate buffers, allowed to stand for 30 minutes in the cold, and then centrifuged. Results are expressed as percentage of nitrogen remaining in solution. Buffers were prepared by mixing varying proportions of $M/15$ Na_2HPO_4 and $1 M$ H_3PO_4 (○); buffers prepared from $M/15$ Na_2HPO_4 and $M/15$ H_3PO_4 (●). Phosphoric acid or tribasic sodium phosphate was used for studying extreme pH values.

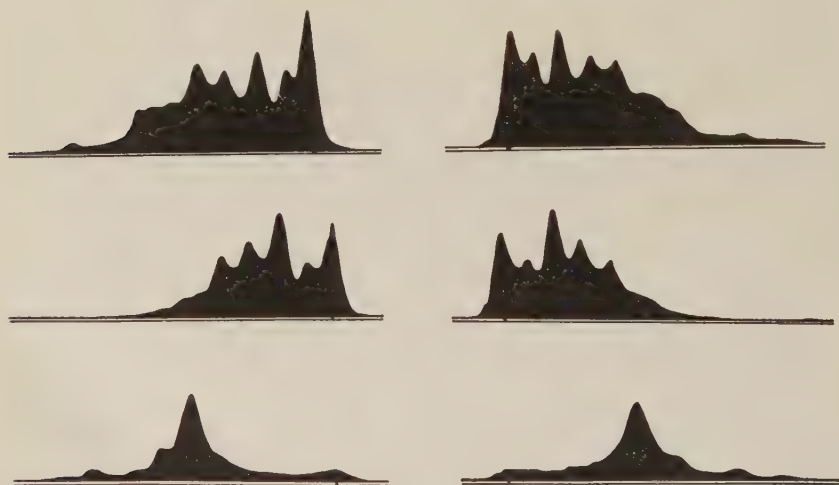


Fig. 5 Precipitation of ratlivsol proteins at pH 5. Top patterns, control; center patterns, protein not precipitated at pH 5; bottom patterns, proteins precipitated at pH 5 after resuspension in pH 7.5 phosphate buffer. All runs made in pH 7.5 phosphate buffer, $\mu = 0.1$ under identical conditions.

increased during the run. When the current was reversed, all components returned to the starting boundary except the precipitate. NaCl added to the buffer eliminated precipitation.

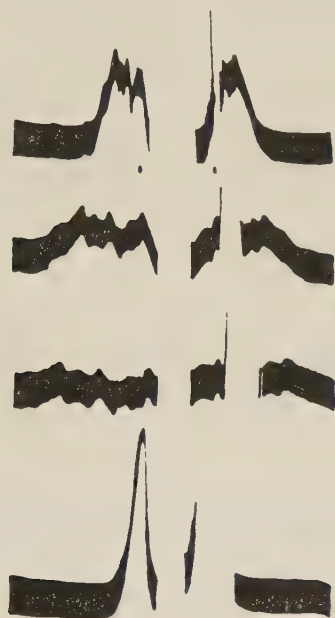


Fig. 6 Electrophoresis of ratlivosol in veronal buffer, pH 8.6, $\mu = 0.1$, containing no NaCl. Ascending limbs on left. As electrophoresis progresses, blank space in descending limb widens. When current is reversed (lower patterns), the material in the ascending limb returns approximately to the starting boundary. In the descending limb, however, the precipitation area (blank space) remains. Note that the precipitate band maintains approximately the same width when the current is reversed but does move backward.

The formation of a precipitate between two peaks in an electrophoretic pattern may be taken as an indication that one constituent complexed and solubilized the other. It appears that the solubilizing molecule has a faster mobility than the less-soluble component. As the former is moved away from the descending starting boundary, the complexes are left behind. As the complex dissociates, the solubilizing molecules are swept away, leaving the less-soluble component to aggre-

gate and precipitate. Similar complexing between large and small molecules is suggested by the finding that some precipitation almost always occurs during dialysis but very little occurs in nondialyzed controls of the same *pH*.

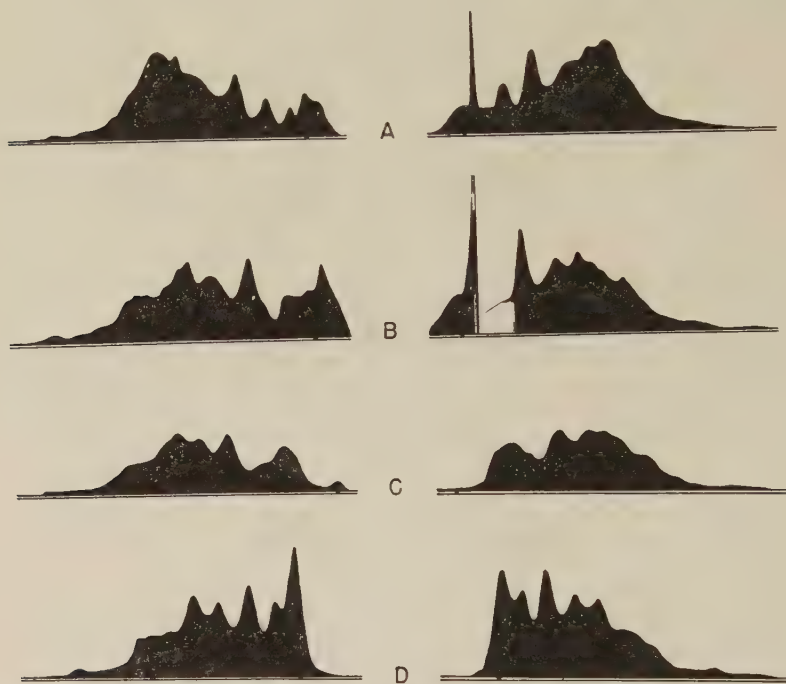


Fig. 7 The effect of *pH* on the electrophoretic pattern of ratlivosol. A, *pH* 9.0, veronal buffer; B, *pH* 8.5, veronal buffer; C, *pH* 8.5, veronal buffer with NaCl; D, *pH* 7.5, phosphate buffer. $\mu = 0.1$. Note the marked changes occurring with changes in *pH*, and at *pH* 8.5 in the presence and absence of NaCl.

Further changes suggestive of complex formation are seen when the effect of *pH* on the electrophoretic pattern of the ratlivosol fraction is studied. As shown in figure 7, very marked differences are present when the patterns observed at *pH* 9.0 and 7.5 are compared. Evidence for several new peaks appears at the higher *pH* and the greater part of the proteins begin to group into one very large broad peak. Noteworthy

also are the differences observed in veronal buffers with and without NaCl.

The effects of freezing on cells have often been attributed to the deleterious effects of high salt concentrations on tissue proteins. When ratlivosol is dialyzed against increasing concentrations of NaCl, only 0.56% of the nondialyzable nitrogen precipitated in 1 *M* NaCl, 6.2% was precipitated in 0.1% *M* NaCl, whereas as much as 42% precipitated in distilled water. Contrary to expectations, therefore, high salt concentrations appear to have a stabilizing effect.

The effects of anesthetics have been studied and will be reported in detail elsewhere. In summary, it may be stated that very low concentrations of ether and alcohol have a marked precipitating effect.

LABILE COMPLEXES AND CELL FUNCTION

In one way or another, the effects observed are caused by interactions of fairly large molecules. The parallels found between the reactions of soluble cytoplasmic proteins and the reactions of living cells are sufficiently suggestive to warrant a consideration of the possible underlying mechanisms. The results thus far obtained are compatible with the concept that certain cytoplasmic colloids form labile complexes that are responsive to a wide variety of conditions, including changes in temperature, ionic strength, ionic composition, dielectric constant, and electrical currents. The following considerations are intended to point out the central role that labile cytoplasmic complexes may play in cell function.

It is evident that a wide variety of colloidal complexes exist in the cell. Nucleohistones may be dissociated and extracted by molar NaCl solutions, as may also a fairly large fraction of microsomal proteins. The proteins of the mitochondria may be solubilized by a variety of detergents. Other complexes require more drastic methods. Proteins believed to be essential components of cellular membranes are solubilized by very alkaline solutions (Dallam, '55). Cells extracted with strong

salts, detergents, and alkaline solutions are not completely dissolved but leave an insoluble residue. These observations show that a variety of types and strengths of bindings hold the cellular building blocks together. Although many of these binding forces are very weak, even weaker forces may exist that produce even more-labile soluble complexes in the cytoplasm. The presence of a sol-gel system in the cytoplasm is sufficient proof of the existence of such labile complexes, even without the experimental data presented here. Such labile aggregates may however have a much wider range of functions than merely changing the consistency of the cell.

Organization of discrete molecules into complex structures comprises one of the central problems of cell physiology; specifically, if proteins are synthesized by the microsomes, how an intramitochondrial protein finds its way there, and how especially the rather insoluble membrane proteins are transported. The most plausible mechanism appears to be via the formation of complexes soluble enough to allow movement by diffusion but labile enough to allow the structure-forming element to dissociate and find its place in a stable molecular fabric.

If structure-forming proteins are present in the rat liver sol system and are kept in solution by virtue of their combination with other more-soluble macromolecules, then it would be expected that sweeping away the solubilizing molecules in an electric field would cause the structure-forming ones to precipitate. This appears to be the simplest explanation of the results actually observed in systems studied electrophoretically in the absence of the chloride ion (which is almost absent from the liver cell), where a precipitate is observed on the cathode (descending) side.

Potential gradients exist across cell membranes and probably across intracellular membranes as well. This potential gradient might produce a precipitation of membrane-forming proteins analogous to that observed in the electrophoresis cell. At first glance, the precipitate appears to be formed in the

wrong limb of the electrophoresis cell, since the cell exterior is generally positive in relation to the interior. The potential would then cause a precipitate to form in the center of the cell and the anodically migrating proteins would tend to move away from the center of the cell. It must be remembered, however, that there are no electrodes inside and outside the cell and that a current is not flowing steadily across the whole cell surface. But a very different condition exists when the cell is injured, as by tearing. A current of injury flows in the opposite direction to the current across the uninjured portions of the membrane. In the area of injury, cellular proteins would generally be moved electrophoretically back into the cell, leaving behind proteins solubilized by loose complex formation. The net effect is to produce a membrane precisely where it is needed — at the point of injury. The mechanism is illustrated in figure 8.

From this point of view, it appears that the cytoplasm contains a system for forming membranes wherever potential gradients exist. Any rapidly metabolizing part of the cell would, therefore, produce around itself a membrane, as may also the chance aggregation of a group of enzymes catalyzing one chain of reactions.

This autoprecipitation mechanism may also provide an explanation for the conflicting results obtained when currents are passed through living cells. Heilbrunn and Daugherty ('39) have concluded that since gelation is observed at the cathode end, cytoplasmic colloids are positively charged. But evidence from a variety of sources uniformly points to the opposite conclusion, namely, a system of negatively charged particles (Anderson, '56a). In the cell, a piling up of protein at the anode side would be expected, but the methods used do not measure the protein concentration. At the cathode side, the insoluble structure-forming proteins would be expected to be left behind, and to cause gelation or precipitation. The latter could well be interpreted as being caused by movement of

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material into this end of the cell, whereas, actually, the opposite is true.

In certain very actively metabolizing cells, concentric rings of membrane-like materials are observed in electron micrographs. This may well be an exaggeration of the autoprecipitation mechanism.



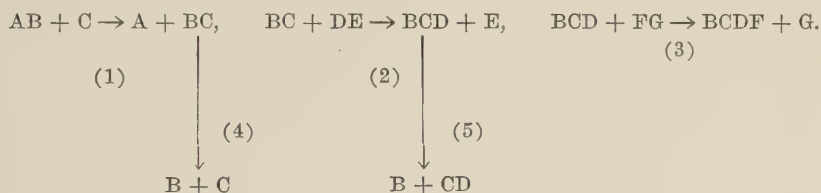
Fig. 8 Schematic drawing of solubilization of relatively insoluble proteins (shown as large particles) by other proteins (shown as small particles). When an electric current is passed through the system as shown on the right, the solubilizing molecules are swept away, leaving the less-soluble molecules to aggregate as shown at top right.

The sensitivity to heating may be explained in terms of the dissociation of cell complexes by thermal agitation with the precipitation of the dissociated material analogous to that proposed during membrane formation. The sensitivity to anesthetics may be similarly explained. Of even greater interest, however, are the possibilities inherent in the properties of labile complexes for the control and modification of cellular activities and responses.

It is evident that the extension of the aggregation process to form long chains of molecules can give rise to large changes in viscosity, and if changes in the distribution of charges over the surface of such chains occur, contraction may result.

This displacement reaction, e.g., the displacement of DNA from nuclei by acid polysaccharides bearing a stronger negative charge (Anderson and Wilbur, '51), may well occur in the cytoplasm. The result may be the formation of new, more-stable complexes and the decomplexing and possible precipitation of a previously complexed substance.

The cellular responses thought to be mediated through the formation of colloidal complexes have been discussed largely in terms of changes in the physical properties of the cell. Complex formation may also produce changes in metabolic patterns. Consider the formation of a substance BCDF from a series of intermediates —



The complete synthesis is catalyzed by enzymes (1), (2), and (3), whereas the intermediates BC and BCD are destroyed by enzymes (4) and (5). It is evident that, if all enzymes and intermediates are free in solution, a good share of the intermediates will be siphoned off. The exact amount will depend on the concentrations of the enzymes and reactants and on the kinetics involved. As the synthetic chain is made longer, the amount of the final product formed will become smaller. If the synthetic enzymes (1), (2), and (3) aggregate, the efficiency with which the final product will be formed will be much increased since each intermediate will form in the immediate neighborhood of the enzyme that catalyzes a further step and

the intermediate-destroying enzymes (4) and (5) will have less chance to act. Although a synthetic chain has been chosen as an example, the same concepts apply to systems catalyzing a stepwise degradation. The formation of aggregates can therefore have a profound effect on the whole rate of a multienzyme reaction.

Enzyme complexes have been observed in a number of instances. In bacterial extracts studied electrophoretically on starch, peroxidase activity was widely distributed. Only after purification by salt fractionation and other methods that would dissociate complexes was the activity limited to a well-defined band (M. I. Dolin, unpublished). The pentose cycle enzymes from rabbit kidney and liver and from a number of other sources sediment together as a complex or microorganelle (Newburgh and Cheldelin, '56). Mitidieri and coworkers ('55) found xanthine dehydrogenase distributed in rat serum in three globulin fractions. Cooper and Lehninger ('56) extracted an enzyme complex from mitochondria, using digitonin, which was capable of oxidative phosphorylation. Cell division with the complete reduplication of all parts suggests that all the structures of cells that can divide are somewhat labile. There would seem to be no other way that a newly synthesized protein, possessing the proper credentials, could work its way into the fabric of the cell.

Lability is a prerequisite for an equilibrium system in which structure is in equilibrium with either dissolved substances or complexes that are in turn in equilibrium with the synthetic mechanism or template. Each state or condition of the protein as it moves from site of synthesis to site of function may be considered as being in a separate compartment in the sense previously discussed (Anderson, '56c).

In summary, therefore, the entire cell is visualized as a system composed of a series of complexes varying widely in size and lability and constituting in their entirety a highly sensitive and responsive equilibrium system.

GENERAL DISCUSSION

WAUGH²: I would like to make a few remarks about an interaction that Dr. Peter von Hippel and I observed in a truly labile colloidal system. This is the system extracted from milk and consisting of two proteins, α_s -casein and a new protein, κ -casein. The latter is the casein that is responsible for stabilizing the colloidal micelles of milk.

α_s -Casein alone at pH 7 forms a colloidal system on its own. κ -Casein is also a colloidal system alone at pH 7. The monomeric units of both are about 15,000 in molecular weight, the colloidal particles consisting of a number of monomers. If these two colloids are mixed in exactly the proportion that they occur in milk a stoichiometric interaction product is developed, an α_s - κ complex, which is also a colloid. If calcium is added to α_s -casein alone, immediately there is a heavy precipitate. Calcium added to the α_s - κ complex immediately forms a set of perfectly stable micelles. If one uses not the normal ratio of α_s -casein to κ -casein as it exists in milk but either an excess of α - or κ -casein, then there forms an α_s - κ complex that utilizes just the correct amounts of the two, and the component that is present in excess forms a separate colloid.

These are interactions of colloidal systems at pH 7. As just noted, when κ -casein is present in excess, the excess material forms a separate set of polymers. In such a system, if the pH is increased to 12.5, where α - and κ -casein exist as monomers, and then the pH is returned to 7, the delicate balance just described has been disturbed completely. A very inhomogeneous distribution of colloidal particles results and one in which there is no apparent excess of κ -casein. The components at the higher pH have not been destroyed, for it is possible to prepare α_s - and κ -caseins from the heterogeneous polymers.

I feel that one must be extremely careful in dealing with labile colloidal systems. Our studies of casein suggest that one must understand the properties of the individual interactants and on this basis formulate an understanding of how the more complicated series of interactions take place.

² David Waugh, Massachusetts Institute of Technology.

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LABILE COLLOIDAL COMPLEXES OF THE CELL¹

A DISCUSSION

PAUL R. GROSS

New York University, New York City

TWO FIGURES

Dr. Anderson's approach to the study of labile colloidal complexes of cells involves homogenizing the living material and then fractionating down as closely as possible to the "soluble phase," where one expects to find such complexes. Our approach (Gross, '54), which also involves homogenization of the cells, requires that we use the homogenate in a condition resembling the cytoplasm as closely as possible, i.e., after separation of as few components as possible in any given procedure. We feel that in this way, although we work with a less well-defined system, we have a better chance to capture *in vitro* the labile colloidal reaction systems known to occur in living cytoplasm. Ultimately, of course, one must isolate and fully characterize the components of the system responsible for the colloidal changes that are elicited in the whole homogenate. I am afraid that at the moment Dr. Anderson's approach is as unlikely to endear him to the physical chemists as mine is to awaken enthusiasm among physiologists.

The imaginative and thorough work reported by Anderson aims largely at the production of a catalogue of "soluble phase" macromolecules prepared each in a mild and reproducible manner. One of the fundamental objectives underlying the work, as I understand it, is an identification of the species of macromolecules responsible for the maintenance and alteration of the physical state of the cytoplasm. On this point I

¹ The author's research is aided by a grant from the American Cancer Society.

should like now to add some information, some of it new, some 2 or 3 years old, that seems of interest in the present discussion. I should like to show that we have, in fact, been able to duplicate in suitably prepared breis certain colloidal (and related metabolic) phenomena long known to occur *in vivo*. Hultin ('50), in Sweden, showed that recalcification of a brei of sea urchin eggs deprived, prior to breakage, of their divalent cations initiates a series of reactions that imitate with surprising fidelity the events that occur after stimulation or fertilization of the normal living cell.

Among the reactions are a sharp increase in the oxygen uptake of the brei, an increase in viscosity, a rapid release of acid, and a pronounced lysis of certain cytoplasmic inclusions. For this discussion, the viscosity increase is of particular interest: it has been considered for many years an indication of extensive reorganization of the cytoplasm *in vivo*; one that characteristically precedes cell division, for example, and *in vivo* appears to be somehow dependent on the presence of Ca^{++} . The importance of Hultin's demonstration of these phenomena *in vitro* cannot be overestimated.

What seems most useful for future investigation in these results is the possibility that a change can be easily produced in the brei, making it more viscous, and in a way that seems related to the *in vivo* mechanism. Hultin was unable to carry this work to the point where changes at the protein level were unequivocally demonstrated, mainly for technical reasons. We were fortunate enough to be able to show (Gross, '54) that this recalcification with small quantities of the ion does, in fact, produce a marked increase in the sedimentability of a fraction of the cytoplasm that is in what would, I suppose, be called the soluble phase.

We have had an opportunity to look at this material directly with the electron microscope as well as to observe its electrophoretic behavior. With certain rather simple methods, we are able to see in the electron microscope microflocs of the aggregating material; we interpret their condition in the wet

state as an extensively cross-linked network of adlineated globular particles, each about 350 Å in diameter. Such a microfloc, chromium shadowed but unfixed, is shown in figure 1. These particles do not aggregate in the + 2-ion-free controls, and can hence be washed off the grids with water along with almost everything else in the homogenate. Analysis of treated

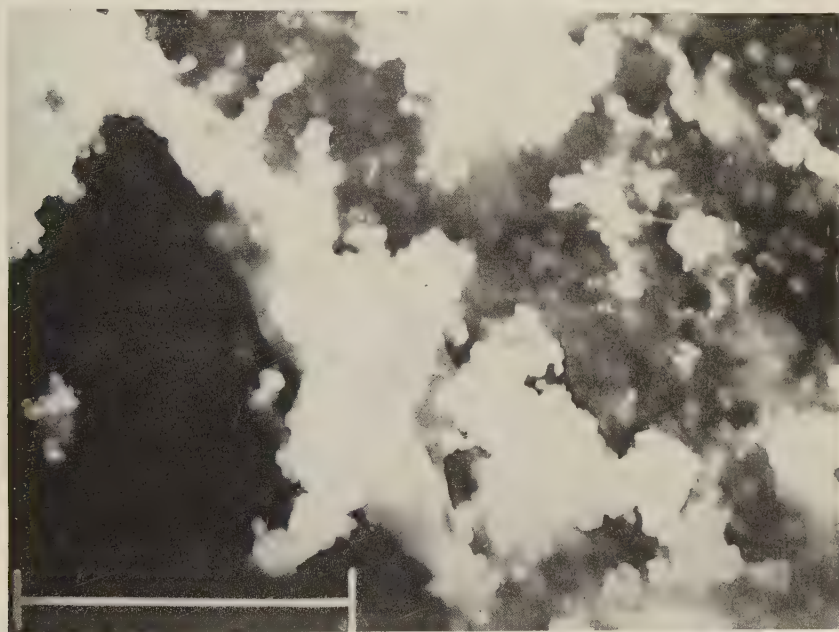


Fig. 1 Electron micrograph of material aggregated in a sea urchin egg homogenate after addition of 0.003 *M* CaCl₂. The line represents a length of 1 μ .

homogenates shows that the aggregating material is rich in pentose nucleic acid (Gross, '56). This does not seem to agree with what Anderson has found.

Our aggregating or polymerizing material may actually be a smaller, soluble phase protein, and these PNA-rich particles ("submicrosomes"?) may simply be occluded in the clot. Although I must be convinced by better data than are currently available that such is the case, it is not difficult to suppose that it might be true, in which case we should still

have to deal with just such a "gel" as I have shown, perhaps not nearly so extensively cross-linked, in living cytoplasm. In the cytoplasm, the PNA-containing particles would be inseparably mixed with the monomer of the small clotting protein, if such a thing exists.

In the sea urchin egg material, our data thus far show that the initiating ion, Ca^{++} , is not bound to the newly sedimentable aggregate, and hence we may not be dealing with a simple double-layer effect. Other observations support the possibility that a limited proteolysis, something like that occurring in fibrinogen clotting, is involved. The problem of Ca^{++} binding to the aggregating macromolecule is one that should receive more satisfactory investigation with isotope methods in the near future. Such a direct approach will have to be essayed, because we have found that in mammalian tissue cells, in which most of the events following recalcification mimic those in the invertebrate egg material, our calcium-binding data (obtained from EDTA titrations) are equivocal; i.e., small amounts of calcium are bound to the sedimenting material, but it is not certain whether the ligand is the aggregating fraction or whether some larger particle fraction is simply absorbing a few divalent cations, and giving up a few protons to the supernatant.

Our experiments (Gross and Pearl, '56) with the mammalian material have been in another regard highly encouraging, for we know now that the constellation of reactions that is established when calcium is released into cytoplasm (or into materials of recent cytoplasmic origin) is of wide distribution in living cells: indeed, if the older physiological literature can be interpreted in this regard, it is almost universal.

Pearl and I have found that if we employ proper perfusion techniques (shown us by Dr. Anderson) we can duplicate in rat liver almost all the reactions that occur in the sea urchin egg brei. As I have already mentioned, the only contradictory result has been obtained in our determinations of calcium binding by sedimenting material, and this is really more an equivocation than a contradiction.

Figure 2 shows the results of two experiments that illustrate the foregoing points. The graphs (whose curves are empirical) show the time course of decrease in solubility (defined here as nonsedimentability at $16,000 \times g$ for 5 minutes) in PNA and in protein in a control and a calcium-treated rat liver brei free of nuclei and whole cells. Plotted against

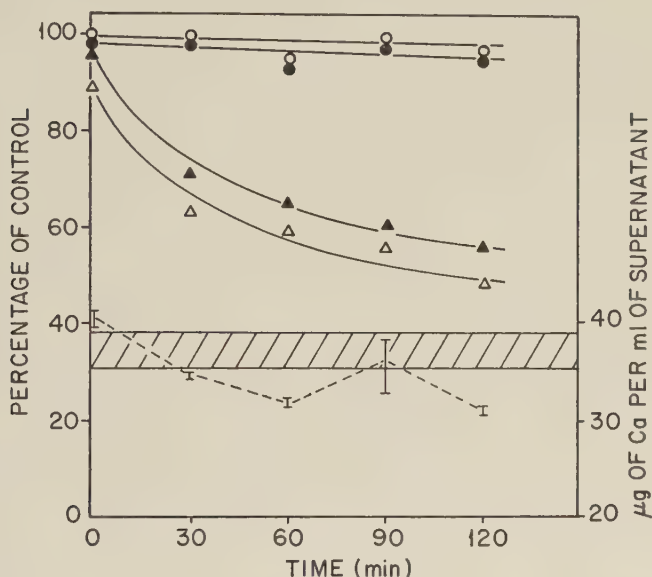


Fig. 2 Time course of precipitation of protein and PNA from rat liver brei after treatment with $0.025 M$ $CaCl_2$, and of loss of calcium from $16,000 \times g$ supernatants. Left ordinate: \circ , control (no calcium) protein; \bullet , control PNA; \blacktriangle , experimental protein; \triangle , experimental PNA. Right ordinate: spread of determinations. Shaded area shows the region of no binding by sediment.

the right-hand ordinate with reference to the same abscissa are data showing analyses of calcium in the supernatants. The vertical lines show the spread of observations at each point: the bar containing slant lines encloses a region in which values should fall were there no binding of calcium to sedimenting material.

The details of these last experiments will appear in a forthcoming publication. I should, perhaps, apologize for present-

ing the data in so hurried a fashion, but within the limits of my function as a commentator on the one hand, and a believer in the importance of the problem, on the other, these remarks have perhaps achieved as concise a form as could be expected.

Permit me then to reiterate: I believe that Dr. Anderson's approach and my own may converge on a method that will enable us to identify the molecular mechanism that underlies the cytoplasmic sol-gel transformation and to find a way rationally to interfere with it. This problem has been avoided in our field for many years, partly because other, more interesting systems (e.g., muscle) were available for the physiologically minded chemist, and partly because suitable methods and rationale have been lacking. Certainly, good protein chemists have avoided thinking about the problem, and this has been a result of the fastidiousness about material that has been the basis of many of the brilliant findings about protein and nucleic acid structure. I wish merely to point out that in our zeal to establish reproducible molecular entities among biopolymers of interest, we may forget that for a satisfying analysis we must know in detail their biological roles and their possibilities for interaction with other biocolloids *in vivo*.

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THE COMPOSITION OF CONTRACTILE MUSCLE PROTEINS

K. LAKI

*National Institute of Arthritis and Metabolic Diseases, National Institutes of
Health, Public Health Service, U.S. Department of Health,
Education, and Welfare, Bethesda, Maryland*

TWO FIGURES

In muscular contraction, a network made of protein molecules and strong enough to support tension interacts with the surrounding medium; as a result of this interaction the network shortens. There is no need to deny that in spite of the vigorous research in this field we do not know what muscular contraction on the molecular level really is. Our ignorance, I believe, arises from our meager knowledge of what the tension-bearing protein network looks like. We have to know more about the "contractile proteins" of the muscle before we can hope to understand their interactions with the surrounding medium.

Of the three fibrous proteins of the muscle fiber, myosin and actin have been demonstrated to be directly involved in contraction; for tropomyosin, only an indirect role has been suggested.

Through the pioneer work of Edsall ('30), von Muralt and Edsall ('30), and Weber ('34), myosin became identified as the contractile protein of muscle. A striking corroboration of those early findings was Szent-Györgyi's demonstration ('41) that threads made of myosin contract when adenosinetriphosphate (ATP) is added to them, provided that the ionic milieu is proper. A great step toward our understanding of this contraction was made when Szent-Györgyi and Banga discovered that there are two kinds of myosin and what had earlier been

called myosin is really a mixture of two proteins. One of these proteins that resembled earlier "myosin" preparations retained the name; the other protein, for which Straub ('42) worked out a method of preparation, was called "actin."

It is important that we decide whether the actomyosin complex or one of the two proteins represents the contractile structure. Some difficulties are encountered when we consider the actomyosin complex as the contractile protein (Kafani and Engelhardt, '53). In solution, ATP breaks the union between myosin and actin. Still, at a low salt concentration, where the shrinkage of the actomyosin gel or the shortening of threads can be observed, the effect of ATP might not be dissociation but some kind of action on the complex to bring about contraction. The electron microscopic studies of Spicer and Rozsa ('53) in this laboratory, however, demonstrated that even under these conditions ATP causes separation of actin from myosin. If not the complex, then one or the other of these two proteins must be the contractile, shape-changing protein. The experiments of Parrish and Mommaerts ('54), Laki *et al.* ('52), Laki and Carroll ('55), and Spicer and Weise ('55) have shown that the behavior of myosin is peculiar. The sedimentation constant of the myosin changes with temperature. It is much higher at room temperature than at 0°C. And this change is reversible within certain limits (Laki and Carroll, '55). The change with temperature may be explained by a change in shape and state of aggregation.

If the myosin is the contracting unit, it would be in the long form at low temperature and in the short form at room temperature (Gergely and Laki, '50; Botts and Morales, '51). Obviously, at room temperature it would be difficult to see shortening of the myosin molecules because they would already be in the shortened form. All one can expect is to see some elongation. This is indeed what Blum and Morales ('53) observe in light-scattering experiments. If we are to see shortening at room temperature upon addition of ATP, we must keep the myosin molecules extended. Possibly the addi-

tion of actin accomplishes this purpose. ATP then causes the dissociation of the myosin, which in turn shortens. Actomyosin in this sense may be the contractile protein of the threads.

It is becoming increasingly apparent that we must know more about ATP and myosin to understand their detailed interaction. Important findings have been reported on the thermodynamics of the so-called "high-energy phosphate" bond in ATP (Kitzinger and Benzinger, '55; Podolsky and Morales, '56).

In this paper, myosin will be considered as the basic structural material of muscular contraction, and the discussion will be centered around it. First, the relation of myosin to tropomyosin and to actin, then the nature of phosphorus in myosin preparations will be examined.

SIMILARITIES BETWEEN MYOSIN AND TROPOMYOSIN

In comparing the amino acid composition of tropomyosin and myosin, Bailey ('48) pointed out the abundance of dicarboxylic acids in the two proteins and suggested that tropomyosin might be a precursor or a subunit of myosin. This idea deserves consideration since, according to recent studies, myosin does not appear to be a homogeneous protein but rather an aggregate of smaller, uneven particles. Urea (Tsao, '53a) breaks myosin to pieces, and even simple dilution of myosin solutions results in the formation of various fragments (Joly *et al.*, '55).

Since attempts to isolate tropomyosin from myosin by the usual methods of tropomyosin preparation have been unsuccessful, an indirect approach was adopted. D. R. Kominz and F. Saad, with the cooperation of the author, isolated tropomyosins from various animals and determined their amino acid composition and some of their physicochemical parameters. These experiments demonstrated that tropomyosin is widely distributed in the animal kingdom. In these investigations, the amino acid composition lacked tryptophan and had a high glutamic acid and low phenylalanine content characteristic of

rabbit tropomyosin. There were, however, definite differences in the relative amounts of certain of the amino acids (see table 1, sect. I for examples).

Since there are tropomyosins of somewhat differing composition, we may compare them with the corresponding myosins and decide whether the idea that tropomyosin is a subunit of myosin can be supported. One way would be to subtract the residues in 10^5 g of tropomyosin from those of myosin (M-TM). For example, in the two proteins from rabbit, a

TABLE 1

Amino acid content of some tropomyosins, and the difference between the amount of amino acids in tropomyosins and the corresponding myosins

	I. TROPOMYOSIN			II. MYOSIN MINUS TROPOMYOSIN		
	Rabbit	Lobster	Fish	Rabbit	Lobster	Fish
	<i>No. of residues per 10^5 g of protein</i>					
Glutamic acid	211	190	185	— 56	— 59	— 65
Proline	1.7	9.5	5	+ 22	+ 17	+ 17
Glycine	12.5	24	19	+ 26	+ 22	+ 22
Alanine	110	86	72	— 32	— 20	— 20
Isoleucine	29	17	24	+ 13	+ 21	+ 21
Phenylalanine	3.5	15	4	+ 23	+ 16	+ 16
Histidine	5.5	3	6.6	+ 9	+ 11	+ 11
Lysine	110	82	102	— 22	— 14	— 13
(The rest of the amino acids exhibited a similar relationship.)						

The amino acid determinations were performed by the method of Moore and Stein ('51). The details of these experiments will be reported elsewhere.

pattern of plus and minus numbers is obtained showing that some amino acids are more abundant in tropomyosin and some in myosin (table 1, sect. II). If the same kind of comparison is made for these two proteins originating from lobster or fish, the same pattern is obtained (table 1, sect. II). This relation may be put this way: Myosin changes from one animal to another in the same way that tropomyosin does. Thus, although these proteins are different, their relation to each other (M-TM) remains constant.

There is no reason why this relation should exist unless tropomyosin indeed constitutes a substantial part of myosin

or is formed according to a pattern similar to myosin. If this relation is taken to indicate that tropomyosin is a component of myosin, then the other component that complements tropomyosin to constitute myosin must remain practically unchanged from one species to the other, as the constancy of M-TM indicates.

A further indication that tropomyosin and myosin are related is provided by experiments of J. Gladner, D. R. Kominz, and the author. These experiments, in which we used carboxypeptidase, revealed that rabbit tropomyosin has 1 mole of isoleucine and 1 mole of serine as the C-terminal groups, whereas tropomyosin from human uterus has 1 leucine and 1 asparagine, as the end groups per mole. (The molecular weight of tropomyosin is between 50,000 and 60,000.) The corresponding end groups in the myosin have been determined only semiquantitatively, but there seems to be no doubt that one of the end groups in rabbit myosin is isoleucine, and that in myosin of human uterus, one is leucine. [According to Locker ('54), 300,000 g of rabbit myosin contains 1 mole of isoleucine as the end group.] If these findings gain confirmation by other end-group methods, they will supply a strong argument that tropomyosin is part of myosin.

SIMILARITIES BETWEEN MYOSIN AND ACTIN

From the amino acid data it is possible to calculate within limits the postulated amount of tropomyosin in myosin. Inspection of the amino acid composition of tropomyosin and myosin immediately shows that tropomyosin cannot be more than 60% of myosin. Also, it must be more than 20% in order to exert its influence on the amino acid composition of myosin. If we assume that 50% of myosin is tropomyosin, we can calculate the amino acid composition of the protein making up the other half of the myosin molecule. Subtraction of the amino acid residues in 10^5 g of tropomyosin from those of 2×10^5 g of myosin gives the composition of the second component of

myosin. Such calculations show that this protein, in contrast to tropomyosin, has a very uniform amino acid composition (see fig. 1). When we determined the amino acid composition of actin we found that these data agreed remarkably with the calculated amino acid composition of the second protein

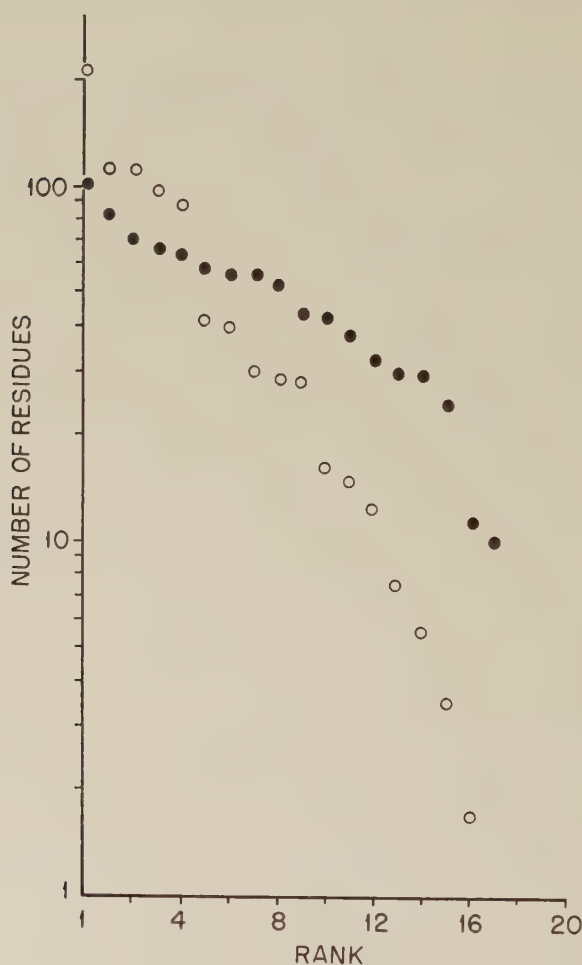


Fig. 1 A comparison of the amino acid composition of tropomyosin and actin. Open circles, tropomyosin; solid circles, actin.

The amino acids are ordered according to their frequency in the corresponding proteins, and against this ordering the log. of the frequencies are plotted. The data show a considerably more even distribution of the amino acids in actin.

(Kominz *et al.*, '54).¹ This agreement shows that, indeed, consideration should be given to the idea that tropomyosin is a subunit of myosin and that the complementary subunit is actin.

Experiments in this laboratory indicate that rabbit myosin contains isoleucine and also phenylalanine as a C-terminal group. Since rabbit actin was found (in agreement with Locker, '54) to contain 1 phenylalanine per $\sim 60,000$ g of protein as a C-terminal group, these findings give further support to the consideration of actin as a component of myosin.

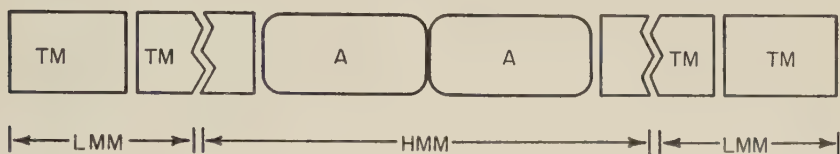


Fig. 2 Schematic representation of the myosin molecule. This figure depicts myosin with a molecular weight of about 420,000; actually the minimum molecular weight could be half of this value ($\sim 200,000$). The molecular weight of actin is taken as $\sim 80,000$ and for tropomyosin as $\sim 50,000$. LMM, L-meromyosin; HMM, H-meromyosin; TM, tropomyosin; A, actin.

THE PROPOSED DISTRIBUTION OF TROPOMYOSIN AND ACTIN IN MYOSIN

The idea that myosin is a compound of tropomyosin and actin will now be examined in more detail. For this purpose, data on meromyosins will be used. Trypsin or chymotrypsin (Gergely, '53; Mihalyi, '53; Mihalyi and Szent-Györgyi, '53; Gergely *et al.*, '55) splits myosin (M wt 420,000 — Laki and Carroll, '55) into three pieces, two light meromyosins (M wt 96,000) and one heavy meromyosin (M wt 232,000) (Szent-Györgyi, '53).

The myosin molecule is thus a compound of two light (L) and one heavy (H) meromyosin put together in some fashion (see fig. 2).

¹ The agreement in phenylalanine is poor. This will be explained later when it is pointed out that quite a large part of phenylalanine in myosin comes from a "third protein."

The meromyosins are split products of myosin and may not strictly be considered as "subunits." But the arguments developed in this paper will indicate that the meromyosins are close to being the subunits. Since both the amino acid composition (Kominz *et al.*, '54) and the molecular weights of the meromyosins are known, tropomyosin as well as actin may be assigned to the meromyosins. In this way we may obtain a more detailed picture for the distribution of tropomyosin and actin.

The argument with these data goes as follows: On the basis of amino acid composition, not more than 32,000 g of intact actin could be subtracted from 1 mole (96,000 g) of L-meromyosin. Since actin is practically not split by trypsin (Mihalyi, '53), and since its molecular weight is greater than 32,000, L-meromyosin cannot contain actin. Therefore, all of the actin supposed to be present in myosin must be found in H-meromyosin.²

Six amino acids (proline, glycine, methionine, tyrosine, histidine, tryptophan) uniformly determine that 1 mole of H-meromyosin can contain about 160,000 g of actin. This would then be the weight of 1, 2, or 3 actin molecules, giving the possible molecular weights of actin as 160,000, 85,000, or 53,000.

If we subtract the amount of actin just found from myosin, the remainder should represent the upper limit of tropomyosin that myosin can contain. Again, a number of amino acids (alanine, methionine, and tyrosine) determine that about 200,000 g of tropomyosin can be subtracted. It is obvious that the figures (160,000 g of actin plus 200,000 g of tropomyosin) so arrived at do not add up to the molecular weight of myosin (420,000 g); some 60,000 g of protein is missing. The conclusion is that there must be a "third protein" in myosin.

² The alternative of subtracting tropomyosin first instead of actin is not considered here because, as will be shown, tropomyosin is split by trypsin.

AN UNUSUAL MUSCLE PROTEIN EXHIBITING THE
PHENYLALANINE SPECTRUM

Calculations show that the amino acid composition of this predicted protein is rather extraordinary. It contains very little tyrosine (up to 5 residues per 10^5 g) and huge amounts of phenylalanine (90 residues per 10^5 g). The phenylalanine content is actually in such an excess that this protein is expected to give the phenylalanine spectrum rather than the usual protein spectrum with a maximum at 280 m μ . In addition, it is to be expected that just as tropomyosin and actin can be isolated from muscle tissue so should this predicted protein be available for isolation.

Henrotte ('55) described the isolation of an apparently small-molecular-weight protein from carp muscle that was very unusual indeed; it gave a phenylalanine spectrum. Kominz and Saad repeated these experiments and were able to confirm Henrotte's findings. From actual amino acid determinations, it was found that this protein contains 95 residues of phenylalanine and 5 residues of tyrosine,³ very close to the predicted values.

A PROPOSED ARRANGEMENT OF THE MEROMYOSINS
IN MYOSIN

The two L-meromyosins cannot contain more than 150,000 g of intact tropomyosin, as the inspection of the amino acid composition shows, but, as was concluded, myosin contains more than this amount. This means that the tryptic splitting must go through some of the tropomyosin regions of myosin so that dissimilar parts of tropomyosin will be located in L- and in H-meromyosin. It will be reported elsewhere in detail that trypsin indeed splits tropomyosin into two fragments about fifty times as fast as it splits myosin.

³ The alanine content of this protein does not fit into our predicted values. Later experiments of Kominz indicate, however, that this protein contains an -S-S-bridge, and the possibility that this connects two chains, one of which fits the postulated amino acid pattern, is being investigated.

With these considerations the meromyosins may be arranged in the myosin molecule. If only a linear structure is considered, the arrangement of the three pieces of the meromyosins could be LLH or LHL. These considerations exclude LLH as a possibility. The L's must be joined, not to each other but separately to the H-meromyosin because they contain tropomyosin, some fragment of which is located in H-meromyosin. All that can be said about the localization of the "third protein" is that it is partly located in H-, partly in L-meromyosin. Since trypsin hardly attacks actin, one would expect the digestion of myosin to stop when the tropomyosin part of myosin is digested away. Indeed, Mihalyi ('53) finds that the splitting of myosin stops when about 50% of it becomes digested. Even more suggestive is the digestion of myosin by a so-called "tropomyosinase." Macfarlane ('55) found that an enzyme from *Clostridium oedematiens*, which preferentially digests tropomyosin to small fragments, digests only half of myosin.

In this picture myosin appears to be a compound of actin and tropomyosin molecules as they exist in salt solution (Tsao, '53b). This point is worth emphasizing since it is becoming increasingly evident that proteins are synthesized from free amino acids and not directly from other proteins (Loftfield and Harris, '56). If myosin is assumed to be synthesized from other proteins such as the meromyosins (Szent-Györgyi, '53) or actin and tropomyosin, it must be a result of an event separate from the primary protein synthesizing action. Then the possibility that myosin is built up on the moment of excitation deserves consideration.

The conclusions arrived at in this discussion actually do not depend on the molecular weights of myosin and H-meromyosin. Calculations in percentages will yield the same conclusions. The only molecular weight that is critical is that of L-meromyosin and it seems well established.

It is hoped that from these experiments some idea will be gained of how the proposed subunits of myosin are held to-

gether; then a rational attempt can be made to find out whether tropomyosin and actin can be isolated from myosin.

THE NATURE OF PHOSPHORUS IN MYOSIN PREPARATIONS

Actin in the globular form contains, fairly strongly bound, 1 mole of ATP to about 60,000 g of protein. According to the picture advanced here, actin is part of the myosin molecule and thus myosin may contain ATP bound to it. Myosin preparations are known to contain phosphorus (Lajtha, '51). We were therefore interested in finding out the nature of this phosphorus in myosin preparations.

Mihalyi and the author found that mild acid hydrolysis [0.6 *M* trichloroacetic acid (TCA) at 60°C.] liberates a mixture of nucleotides and free bases from myosin. By paper chromatography they were identified as adenine, guanine, and cytidylic and uridylic acids.

At the same time, ribose and inorganic phosphate also appear in the hydrolyzate, together with small amounts of amino acids and small peptides. The analysis of the TCA extracts shows that 10⁵ g of myosin contains 1.81 moles of total

TABLE 2

Distribution of purines and pyrimidines in myosin and in the ribonucleic acid isolated from myosin

PREPARATION	ADENINE	GUANINE	CYTOSINE	URACIL
	%	%	%	%
TCA extract	19.0	29.8	32.7	18.9
RNA, NaCl extract, dialyzed	18.0	30.9	31.5	19.4
RNA, NaCl extract, EtOH precipitate	18.9	32.3	29.5	19.1
RNA, NaCl extract, EtOH precipitate	17.2	31.1	32.6	18.9

EtOH = ethyl alcohol.

The determination of the bases was performed by the methods of Kerr *et al.* ('49) and Loring *et al.* ('52).

TABLE 3

The nucleic acid content of myosin preparations

MYOSIN A PREPARATION	PERCENTAGE OF RNA IN MYOSIN	PERCENTAGE OF NUCLEIC ACID P IN MYOSIN
Twice pptd by dilution	0.541 ± 0.086^a	0.051 ± 0.008
Twice pptd by dilution, ultracentrifuged	0.430 ± 0.068	0.041 ± 0.006
Twice pptd by dilution, and twice pptd by (NH ₄) ₂ SO ₄	0.434 ± 0.103	0.041 ± 0.009

^a Deviations from the mean of six determinations.

phosphorus, 1.55 moles of total base, and 0.26 mole of non-nucleotide phosphorus.

The kinetics of the liberation of bases, nucleotides, and ribose is of the first order, similar to that of the hydrolysis of yeast ribonucleic acid. Indeed nucleic acid can be extracted from myosin with hot 10% NaCl solution. From this solution, 60–68% of the material can be precipitated with 2 volumes of ethanol.

In the ultracentrifuge the original NaCl extracts showed a very pronounced heterogeneity, with a mean sedimentation constant of 9.1. The amount of bases present in the nucleic acid corresponds to that extracted by the acid hydrolysis. The ratio of phosphorus to base was 1:1 in all the preparations, and they contained only ribose as the sugar moiety.

Myosin cannot be freed of the nucleic acid by reprecipitations changing the ionic strength, and only partially by ammonium sulfate reprecipitations. However, treatment with ribonuclease (73–88% of the total nucleotides is released by RNase), followed by reprecipitations, removes most of the nucleic acid content of myosin. The myosin thus obtained has the same physicochemical and enzymic properties as the original one. From these experiments it appears that two-thirds of the phosphorus that can be demonstrated in myosin preparations is located in the nucleic acid. If myosin contains actin, it is then devoid of ATP or ADP (adenosinediphos-

phate). The nature of the rest of the phosphorus is under investigation.

DISCUSSION

The structure presented in this paper for myosin is obviously tentative, but it points out the possibility that myosin may be formed outside the primary protein-manufacturing centers from the meromyosins or actin and tropomyosin as its ingredients. Whether this assemblage of myosin occurs on the moment of excitation or at some other instant, a mechanism should exist to accomplish this combination of ingredients.

Thus, even with a perfectly functioning protein-synthesizing system, the assemblage of myosin may fail.

If we consider myosin as being formed on the moment of excitation, the sol state of the protoplasm would become a contractile gel on excitation. Watanabe *et al.* ('53) has already considered the possibility that on excitation the contractile structure is built up, perhaps by utilizing the energy of ATP. This building up the network (sol \rightarrow gel change) may coincide with the "activation heat," though it is conceivable that muscles differ in the extent to which the network preexists. The "state of readiness" for contraction may be different in cross-striated and in smooth muscle.

If on excitation a sol-gel change operates to build up the contractile network, the removal of a contracting agent (ATP, e.g.) or the disruption of the network could bring about relaxation. A failure in disaggregation also could lead to serious defects in the mechanism of muscular contraction. Myosin is known to aggregate on standing, and this aggregation eventually becomes irreversible (Laki and Carroll, '55). On the bases presented in this paper, myosin monomer may be expected to aggregate by virtue of its being composed of tropomyosin and actin — both known to aggregate. Therefore, there may be two types of myosin aggregations: one through the tropomyosin component and one through the actin component. In this connection the findings of Stern *et al.* ('56) are interesting.

These authors found that myosin from the failing heart of a dog has a much larger molecular weight than myosin from a normal heart.

Although we found that the nucleic acid associated with myosin has nothing to do with the enzymic or hydrodynamic properties of myosin, we cannot exclude the possibility that nucleic acid has a role in assembling the myosin from its protein ingredients. The isolation of a tropomyosin also closely associated with nucleic acid (Hamoir, '51) again points to such a possibility.

SUMMARY

A comparison of the amino acid composition of tropomyosin and actin shows that myosin and the meromyosins might be looked upon as compounds of tropomyosin and actin. This assumption predicts, among other things, the existence of a protein with a phenylalanine spectrum.

A small amount of phosphorus usually accompanying myosin preparation was found to be part of a nucleic acid contamination.

ADDENDUM

Velick ('56) supplies the most direct support yet in favor of our proposed composition of myosin.

We have pointed out that six amino acids determine that about 70% of H-meromyosin would be actin. One of those six amino acids is tyrosine.

According to the amino acid analysis (Kominz *et al.*, '54) 232,000 g (1 mole) of H-meromyosin contains 48-49 residues of tyrosine and 93 residues of phenylalanine. About 155,000 g of actin contains 48 residues of tyrosine and 44 residues of phenylalanine. The conclusion that this amount of actin is part of H-meromyosin means that all of the tyrosine (but not phenylalanine, e.g.) in H-meromyosin should come from the actin component.

This conclusion can be checked with labeling experiments. By feeding labeled amino acids to an animal, one ought to find that after isolating the proteins, the specific activity of tyrosine (but not phenylalanine, e.g.) in H-meromyosin (which is a large fragment of myosin), instead of being the same as in myosin, should be the same as in actin. This conclusion, permitting unequal labeling in a muscle protein, is a striking one because it is contrary to the finding that muscle proteins exhibit uniform labeling.

Velick finds that the specific activity of tyrosine in myosin is 2.9 (counts per minute per micromole), in actin 1.2, and 1.0 in H-meromyosin. Considering that the errors in these experiments amount to about $\pm 8\%$, it can be stated that tyrosine in H-meromyosin, instead of showing the activity found in myosin, shows the

activity found in actin. These experiments thus supply one of the strongest arguments in favor of the picture proposed above.

Velick's data can also be used in a less direct way. According to our picture, myosin is composed of a "third protein" together with actin and tropomyosin. The specific activities of the tyrosine (or any other amino acid) in these three proteins should add up to the specific activity in myosin. From Velick's data it is evident that the activities of actin and tropomyosin run somewhat short of the activity of tyrosine in myosin, showing that these two proteins are not the only constituents of myosin.

Unfortunately, amino acid determinations are not sensitive enough for exact predictions of how much tyrosine the "third protein" should contain, but it cannot amount to more than a few residues. One can then calculate from Velick's data that the turnover rate of this small-molecular-weight "third protein" would be two or three times as great as that of myosin.

Some further checks can be obtained from labeling experiments. Since most of H-meromyosin is supposed to be actin, the half life of H-meromyosin should be close to that of actin. On the other hand, the half life of L-meromyosin should be closer to that of tropomyosin. Velick finds the half life of H-meromyosin to be 80 days, of actin 67 days, of L-meromyosin 20, and tropomyosin 27 days. These values are indeed in excellent agreement with the predictions.

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ADENOSINETRIPHOSPHATE AND THE SHORTENING OF MUSCULAR MODELS

WILLIAM J. BOWEN

*National Institute of Arthritis and Metabolic Diseases, National Institutes
of Health, Public Health Service, U. S. Department of Health,
Education, and Welfare, Bethesda, Maryland*

FIVE FIGURES

INTRODUCTION

Myosin has been recognized for nearly a century as a protein that is closely associated with the contraction of muscle, and adenosinetriphosphate (ATP) has been known as an energy-containing constituent of muscle since 1928, when Lohmann described its isolation. In 1939 Engelhardt and Ljubimowa reported that myosin dephosphorylates (splits) ATP. Myosin, therefore, acts as an enzyme (ATPase) and as a contractile protein. When concentrated myosin (natural actomyosin, or myosin B) is extruded into heavy-metal-free water, it forms threads that contract in the presence of ATP (Szent-Györgyi, '41). Later, Szent-Györgyi ('51) found that muscle from which water-soluble compounds are extracted with 50% glycerol solution will contract when the glycerol is removed and ATP is applied. When the psoas muscle is extracted at a fixed length, small straight bundles of fibers that are highly adapted to the study of the action of ATP can be removed. Study of the thread and fiber muscle models has been pursued actively in the hope that the comprehension of the events of shortening in these simplified systems would lead to an understanding of muscular contraction.

The experimental investigation of the contraction of these models and of the ATPase activity of myosin has produced results that have led to at least two interpretations of the role of ATP in the contraction. One interpretation is that muscular contraction is caused by a product of ATP hydrolysis, splitting and contraction being rigidly coupled. The other is that contraction is caused by intact ATP (or other substance sharing its critical properties, e.g., negative charge), with contraction not necessarily linked to splitting. The word, "interpretation," is apt because there are no longer any major areas of experimental disagreement.

The concept that contraction and splitting are coupled was suggested by the parallelism observed between the intensity or rapidity of contraction of models and the rate of splitting of ATP induced by increases in temperature, increases in ionic strength from those near zero, and addition of certain inhibitors, such as fuadin and salyrgan (see Weber and Portzehl, '54). Also, both reactions vary in a parallel manner with additions of ATP if high concentrations (0.01 *M*) are avoided. However, there are also notable instances of nonparallelism between the rates of splitting and shortening, and these support the hypothesis that shortening of the myosin molecule is not dependent on the splitting of ATP.

THE EFFECTS OF POTASSIUM, CALCIUM, AND MAGNESIUM

One instance of nonparallelism occurs when magnesium or calcium is added to the respective solutions of ATP in which shortening of myosin threads and splitting of ATP by myosin are observed. This is illustrated in figures 1 and 2, and table 1. Figure 1 shows that the addition of magnesium accelerates and the addition of calcium inhibits the rate of shortening of myosin B threads (Bowen, '51). Table 1 shows that similar addition of magnesium to mixtures of myosin and ATP had little or no effect on the rate of dephosphorylation but that the addition of calcium quadrupled the rate (Bowen, '51).

Subsequent experiments in this and other laboratories (Hasselbach, '52) under more carefully controlled conditions and with $0.001\text{ }M$ ATP show (vide infra, fig. 4, 5) that $0.01\text{ }M$ MgCl_2 actually inhibits the ATPase activity of myosin B even more strongly than it did in the early experiments. Still

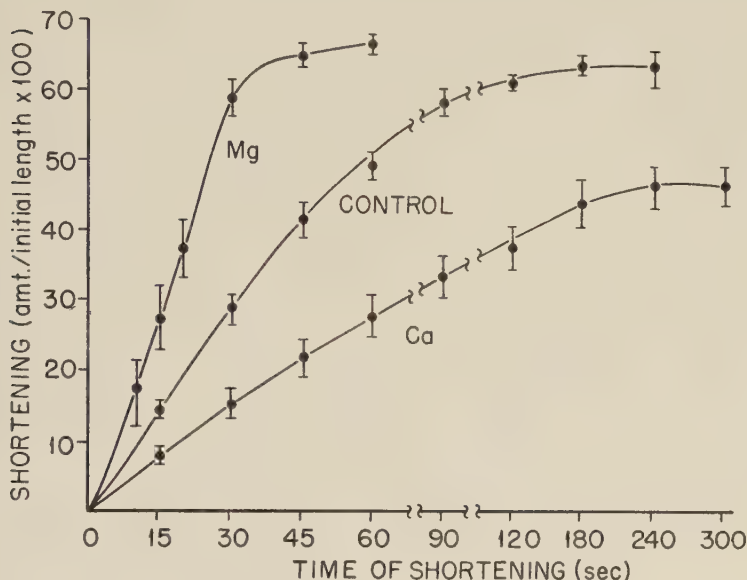


Fig. 1 The effect of $0.01\text{ }M$ CaCl_2 and $0.01\text{ }M$ MgCl_2 on the shortening of myosin B threads in the presence of $6.4\text{--}6.6\text{ }\mu M$ ATP. Ionic strength: 0.12. Shortening: initial length minus the length at time of measurement. Estimated standard error of the average values obtained from measurements of 15 or 16 threads. Measurements were made with the aid of a microscope equipped with a camera lucida.

other experiments demonstrate the dramatic effect of magnesium on the process of shortening of myosin B threads. Shortening was initiated in the absence of magnesium (fig. 2) by the application of ATP. After the rate had been established or had begun to decrease, the solution was made $0.003\text{ }M$ with respect to MgCl_2 . All rates increased immediately upon this addition. This increase in rapidity of shortening is quite different from the effect of $0.003\text{ }M$ MgCl_2 on

dephosphorylation of ATP by myosin B in 0.1 M KCl (fig. 4). Watanabe ('55) found the same effect by adding magnesium during the shortening of bundles of extracted psoas muscle. When CaCl_2 is added during shortening of threads, the rate of shortening is reduced (Bowen, '52).

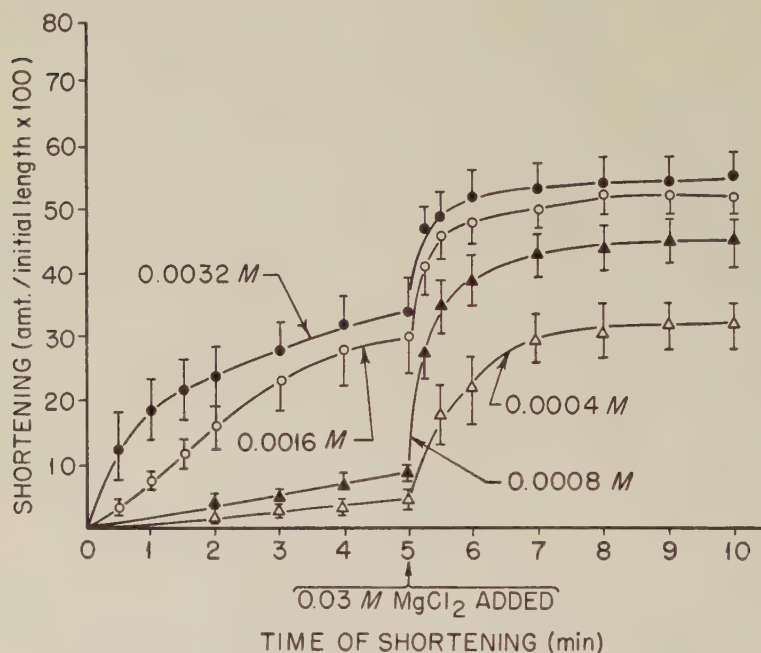


Fig. 2 The effect of four concentrations of ATP before and after the addition of 0.03 M MgCl_2 (final concentration, 0.003 M) on the rapidity of shortening of myosin B threads. Estimated standard error given for each average value.

TABLE 1

Three identical tests of phosphorylytic potency of myosin B

	μG OF INORGANIC PHOSPHATE SPLIT IN:		
	Test 1	Test 2	Test 3
Control	4.4	5.0	5.5
0.01 M MgCl_2	4.6	4.5	4.9
0.01 M CaCl_2	16.5	18.4	14.9

Each 3.0 ml of reaction mixture contained 52 μg of labile phosphate. Each mixture was 0.11 ionic strength. Incubations were 10-minute duration. Dephosphorylation was measured by the Fiske-Subbarow method of phosphate analysis.

These examples of noncorrelation of splitting and shortening were obtained with myosin B suspensions and threads. Others have been obtained with bundles of glycerol-extracted rabbit psoas muscle fibers. At $0.001\text{ }M$ MgCl_2 , the ATPase activity of homogenized glycerol-extracted psoas is depressed by increases in concentration of KCl from 0.02 to $0.3\text{ }M$, but the rate of shortening rises (fig. 3) (Bowen and Kerwin, '55). Increases of MgCl_2 at constant concentrations of KCl have

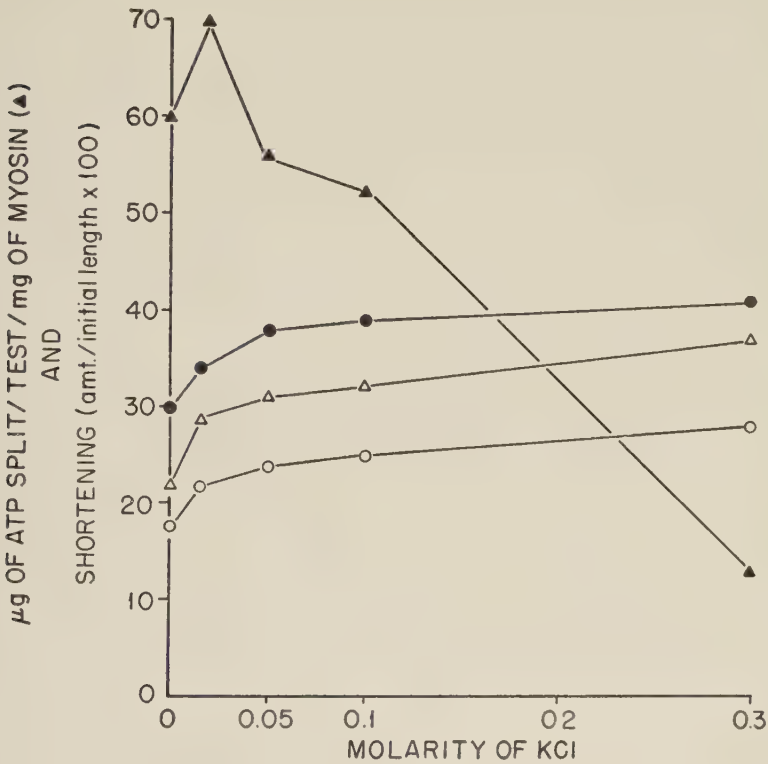


Fig. 3 The nonparallelism of the rates of shortening of extracted psoas muscle fiber bundles and dephosphorylation of ATP by homogenized extracted psoas muscle. Variation of the rates was caused by increasing the concentration of KCl in the presence of $0.001\text{ }M$ MgCl_2 and $0.02\text{ }M$ Tris buffer, $\text{pH } 7.5$. Amount of shortening as in figure 1. Each point on the shortening plot is an average of records from 8 fibers. Percentage shortening after 15 seconds (○), 30 seconds (△), and 60 seconds (●).

demonstrated similar divergences of effect on shortening of fiber bundles and splitting of ATP by myosin. At $0.1\text{ }M$ KCl , increase of $MgCl_2$ from 0 to $10^{-3}\text{ }M$ causes first an increase and then a decrease in the rate of dephosphorylation (fig. 4). At $0.3\text{ }M$ KCl , increase of the $MgCl_2$ to $10^{-4}\text{ }M$ causes only a decrease in the rate of dephosphorylation (fig. 5). At both 0.1 and $0.3\text{ }M$ KCl , however, the rate of shortening was increased only by increasing the $MgCl_2$ over this range of concentrations. It is to be noted that if these observations had been confined to the concentrations of $MgCl_2$ of 10^{-3}

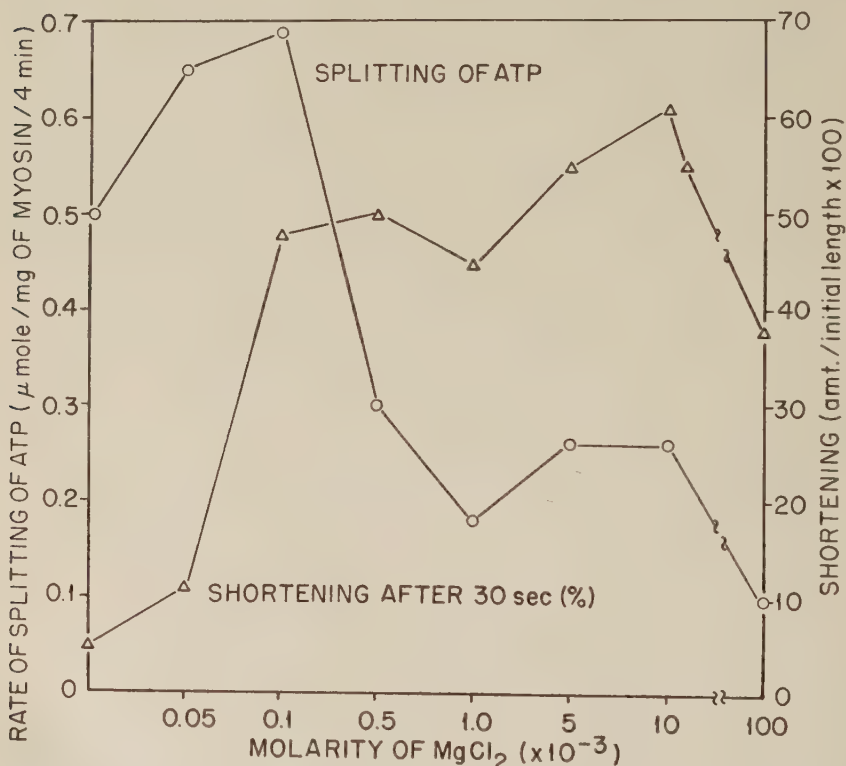


Fig. 4 The nonparallelism of the rates of shortening of extracted psoas muscle fiber bundles and dephosphorylation by myosin B caused by increasing the concentration of $MgCl_2$ from 0 to $0.1\text{ }M$ in the presence of $0.1\text{ }M$ KCl . Fiber shortening was observed on a microscope slide placed over a millimeter scale. Dephosphorylation was measured by the Fiske-Subbarow method of phosphate analysis.

to $10^{-1} M$, there would appear to be only a correlation between the rates of shortening and of splitting, but study of both phenomena at concentrations of $MgCl_2$ from 0 to $10^{-3} M$ reveals a conspicuous noncorrelation.

STEADY-STATE LENGTHS

Another means of studying the role of ATP upon the shortening of extracted bundles of psoas muscle has been the determination of the steady-state length of bundles in various concentrations of ATP (Bowen, '54). Three bundles of fibers of known length were put into buffered (pH 7.0) solutions of ATP of several concentrations and allowed to

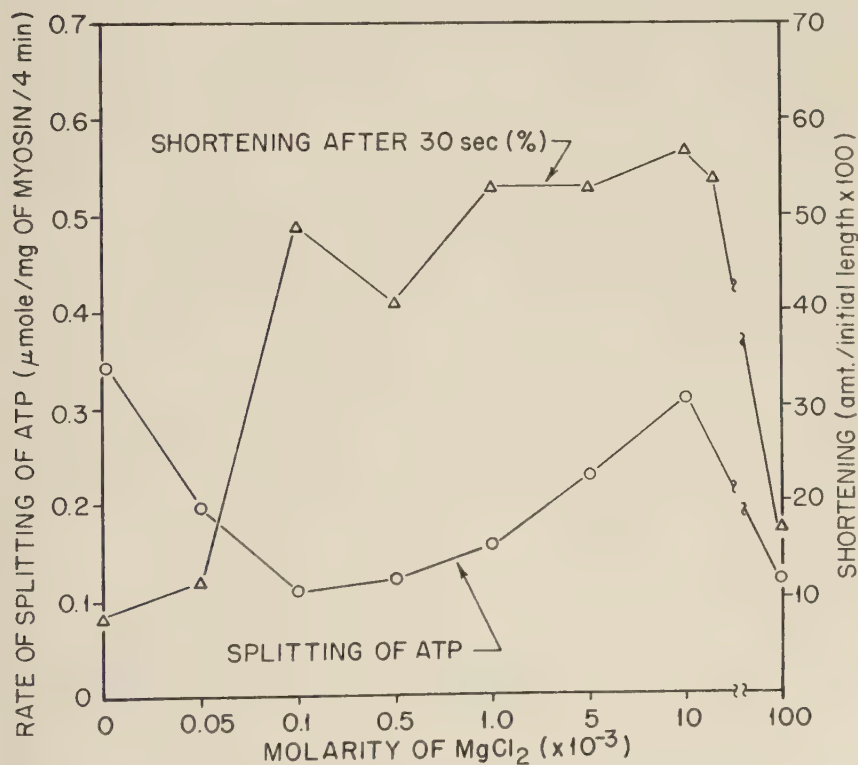


Fig. 5 As for figure 4 except in the presence of $0.3 M$ KCl.

shorten for 15 minutes. The straight lengths were then measured and the bundles returned to the ATP solution. This procedure was repeated until the measurements indicated that shortening had stopped (table 2). Then fresh solution of ATP of the same concentration was added and measurements were made 5 and 20 minutes later. The lengths at those times were directly related to the concentration of ATP rather than to the continuation of ATP splitting. (It has been shown — Bowen, unpublished observations — that fibers that have shortened to a steady state for the concentration of

TABLE 2

Effect of changing the concentration of ATP on the length of glycerol-extracted rabbit psoas fibers

MOLARITY OF ATP →	0.0037	0.0015	0.00075	0.00037
	Mean length of three fiber bundles (mm)			
Before ATP	38.6	40.3	43.0	37.8
After 15 min of ATP	10.0	14.6	26.6	26
After 30 min of ATP	9.6	13.5	25.6	26.6
After 45 min of ATP	9.6 (75)	14.3 (64)	25.6 (41)	27.6 (30)
5 min after addition of fresh ATP	9.6	13.3	23.6	26.3
20 min after addition of fresh ATP	9.6 (75)	13.3 (67)	23.6 (45)	26.6 (32)
5 min after addition of 0.0037 M ATP	..	10.6 (74)	14.6 (66)	11 (71)

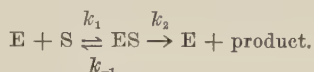
Percentage shortening in parentheses.

ATP continue to split ATP.) The fibers, however, were quite able to resume shortening, as was shown by immersing them in a higher concentration of ATP (table 2).

The correlation of steady-state length with concentration of ATP has been verified innumerable times in further experimentation designed to gather data for estimating Michaelis-Menten constants of the ATP-extracted muscle system (Blum *et al.*, '57). Blum and coworkers found correlation with concentration of ATP at each of several concentrations of KCl and of MgCl₂ and with six widths of fiber bundles.

DISCUSSION

The results of these experiments are not consistent with the contention that the dephosphorylation of ATP *per se* induces shortening of muscle models (Weber and Portzehl, '54). Some other reaction is indicated. Morales and Botts ('52) have suggested the binding of ATP to myosin as the reaction that induces this shortening. Their hypothesis, highly developed in the review by Morales *et al.* ('55), is based on the Michaelis-Menten scheme of reaction of an enzyme (E) with a substrate (S) to give enzyme-substrate complex ES



In this scheme, ES is a complex of myosin and ATP that is considered to shorten prior to decomposition of ATP. The k_1 reaction is necessary for the formation of complex, and both k_1 and k_2 are required for formation of product. Explanation of the diverse effects of cations on shortening and dephosphorylation may lie in this scheme. For example, if k_1 and k_2 are made larger in relation to k_{-1} by increases of the concentration of MgCl_2 from zero to low concentration, then the augmentative effects on both shortening and splitting between 0 and 0.1 *M* MgCl_2 (fig. 4) are logical. Support for this conception has been found in light scattering and ATPase studies of myosin by Tonomura *et al.* ('53). The nonparallel effects of further additions of MgCl_2 may be because the reaction that forms complex is augmented and the one that forms product is inhibited. At 0.3 *M* KCl (fig. 5) increases of MgCl_2 from 0 to 10^{-4} *M* would, according to the scheme, increase k_1 in relation to k_{-1} and k_2 and shortening would be accelerated while dephosphorylation is inhibited. In this instance magnesium would increase the association of ATP with myosin, as has been found by Blum ('55) at 0.6 *M* KCl.

The difference in the effect of magnesium at 0.1 and 0.3 *M* KCl suggests a competition between potassium ions and magnesium ions for the same site. At low concentrations of MgCl_2 , the inhibiting potassium ions dominate. At high concentrations

of $MgCl_2$, the rates of dephosphorylation rise, thereby suggesting that magnesium can replace potassium and that magnesium favors dephosphorylation if there is no interference by potassium. The possibility that magnesium, when not antagonized by potassium, favors dephosphorylation as well as shortening does not deny the contention that the splitting of ATP is unrelated to shortening of muscle models. Such ionic conditions as those presented in figures 1, 3, 4, and 5 cause non-parallelism of the two phenomena; if shortening were rigidly dependent on the splitting of ATP, then the two phenomena should be similarly influenced under *all* conditions.

A logical interpretation of the action of calcium in the Michaelis-Menten scheme is that calcium promotes the breakdown of complex to enzyme and product, thus reducing the concentration of the complex with myosin that deforms to the shortened fiber or molecule.

Results of the experiments on steady-state lengths are also elucidated by application of the Michaelis-Menten concept of enzyme action. By this concept, the extent of shortening is a function of the amount of ATP included in the enzyme-substrate complex, myosin-ATP, or ES, which in turn is a function of the concentrations of myosin and ATP. The concentration of myosin is unalterable in extracted fibers but the concentration of ATP can be varied, which causes the predictable variation in concentration of ES that is manifest by the lengths of the fibers at the termination of shortening. In the experiments with low concentration of ATP, splitting continues until the ATP is depleted. If the energy supplied by this splitting were the only requirement, shortening in low concentrations would be equal to that in high concentrations except that a proportionately longer time would be required to attain it. In the experiments by Blum *et al.* ('57) in which the ATP solution was exchanged for fresh solution at 30-minute intervals, longer times were required for the fibers to reach a steady state, but the length at which shortening ceased was considerably greater in low concentrations than in high.

Therefore, the idea that the extent of shortening is related to the total pyrophosphate-bond energy released is not supported by these results. The concept that the extent of shortening is related to the rate of splitting of ATP, which varies directly with the concentration of ATP, is not opposed by such data as are in table 2. It is opposed, however, by the effects of calcium on shortening and splitting (fig. 1 and table 1).

Thus the nonparallel effects of potassium, calcium, and magnesium on the shortening of muscle models and the splitting of ATP and the steady-state lengths attained in ATP solutions are compatible with the theory of Morales and Botts ('52) that it is the binding of ATP to myosin that causes contraction of muscle. The "binding" theory has been strengthened by the findings from several other experimental approaches (see Morales, '56). These include the finding that iodide or thiocyanate anion can substitute for the anion of ATP in muscle models (Laki and Bowen, '55). Solutions of HgI_2 in KI or KSCN cause 50% shortening and small development of tension of thread or fiber models within seconds after application. Analysis of the extent of shortening caused by KI- HgI_2 solution indicates that the binding constant of ATP is 2000 times as great as that of the iodides (Bowen and Laki, '56); thus ionic strengths of the iodides, in order to cause contraction, must be 2000 times as great as that of ATP. Such high ionic strengths probably account for the smallness of the tension developed by fiber models when treated with KI- HgI_2 solution although iodide ion specificity may contribute. The tensions developed are about one-tenth those developed by the application of ATP, and, with 0.5 *M* HgI_2 -1.0 *M* KI, they relax immediately because of the disintegration in the fiber caused by the high ionic strength. With lower concentrations of KI- HgI_2 solution that cause shortening (see Laki and Bowen, '55), the tensions are maintained, probably because the disintegration of the fiber is less than in 0.5 *M* HgI_2 -1.0 *M* KI.

A further and tentative complexity in the Michaelis-Menten explanation of the events that occur between myosin and ATP is a possible specificity of sites that are involved in shortening and splitting of ATP. For example, there may be two kinds of sites, numbers 1 and 2, only one (number 1 hereinafter) of which need be occupied by ATP to produce shortening, but both of which are involved in splitting. The sites must be made up of interrelated ionizing groups of myosin, because salyrgan (an inorganic mercurial compound) completely inhibits both splitting and shortening (Weber and Portzehl, '54). This shows that bond -SH groups are involved in all sites having to do with splitting and shortening. Less-toxic metallic ions such as potassium, magnesium, and calcium do not block the sites, except at great concentration, and they affect the sites in different manners. The accelerating action of calcium on splitting, and not on shortening, indicates that the splitting site contains a carboxylate group (see Katchalsky, '54, p. 43). Low concentrations of potassium ion ($\approx 0.1 M$) augment the binding of ATP at both kinds of sites and both splitting and shortening are accelerated. High concentrations of potassium ion ($> 0.1 M$) partially block number 2 sites and splitting is inhibited. The same concentration of potassium ion does not interfere with number 1 sites, as evidenced by no interference of shortening at concentrations up to $0.3 M$ KCl (fig. 3). Magnesium ions successfully block number 2 sites at concentrations as low as $10^{-4} M$ in the presence of potassium ion but number 1 sites gain and retain affinity for ATP as the concentration of magnesium is increased. Thus splitting is inhibited and shortening augmented by 10^{-3} to $10^{-4} M$ $MgCl_2$ in 0.1 and $0.3 M$ KCl (fig. 4, 5). Finally, both sites are blocked by concentrations of $MgCl_2$ of 0.15 – $0.2 M$ (unpublished observations); however, inhibition of shortening by a high concentration of $MgCl_2$ appears only above concentrations of $0.1 M$ whereas the inhibition of splitting by homogenized glycerol-extracted psoas is evident at concentrations as low as $10^{-4} M$ $MgCl_2$.

SUMMARY

Several ionic conditions exist that accelerate shortening of muscular models and inhibit the rate of splitting ATP. The extent of shortening of these models is directly related to the concentration of ATP. These results contradict the concept that the splitting of ATP must precede the contraction of muscle to supply the required kinetic energy but they uphold the hypothesis that contraction occurs because myosin binds ATP. The extensive and quick shortening of thread and fiber models caused by iodide and thiocyanate ions also supports the binding theory.

GENERAL DISCUSSION

ANDREW SZENT-GYÖRGYI¹: I wonder to what extent one should be impressed by experiments that show that there are conditions in which enzymic activities and contractility do not go parallel. For instance myosin, free of actin or heavy meromyosin, is ATPase and is not contractile. I think it is more relevant that whenever ATPase activity is destroyed by some means, contractility is always lost. If only binding of ATP was necessary for contraction and not its splitting one would expect to find a preparation that was contractile though its ATPase properties were destroyed.

A second point is that I do not think that shortening caused by potassium iodide mercuric iodide is comparable with the contraction caused by ATP. The tension developed in potassium iodide mercuric iodide is very small, if there is any. It does not show the phenomena of "quick stretch" and "quick release," which are characteristic of the physiological contraction of muscle and to the one induced by ATP.

A third question that worries me considerably in thinking about the "binding theory" concerns the amount of ATP that must be bound in order to account for the work performed in contraction. In such a theory, the difference in the binding energy between ATP and ADP to actomyosin is the ultimate

¹ Andrew Szent-Györgyi, Marine Biological Laboratory, Woods Hole.

energy source, or possibly the difference between ATP-free protein and protein on which ATP is bound. Unfortunately, we do not know the binding constants. Still J. J. Blum found a considerable inhibition of ATPase activity by ADP, indicating that the difference in binding energies is not much more than a factor of ten or so. We would expect that about 1 mole of ATP should be bound by 10,000 g of protein since we have experiments showing that this amount of actomyosin can perform 1000–2000 calories of work. If I remember well, there is only one ATP bound by 100,000–300,000 g of protein from enzymic and light-scattering studies.

BOWEN: I would like to take these remarks as comments, but I gather from the first one that you would like to see splitting of ATP entirely inhibited while shortening is not inhibited. Is that right, Dr. Szent-Györgyi?

SZENT-GYÖRGYI: That is what I would accept as a proof of the contention that binding of ATP is all that is required for contraction.

BOWEN: That would, indeed, be proof and we are fully cognizant of its desirability. We have not given up trying to find such instances. The nearest we have come to it is in experiments with high concentrations of $MgCl_2$, using homogenized psoas muscle as enzyme. When the concentration of $MgCl_2$ was made 0.15 *M*, or greater, we were able to detect no splitting of ATP after 6 minutes, but fine bundles of psoas muscle still shortened, in the first 5 seconds, 25% of the extent that they shortened at lower concentrations of $MgCl_2$. However, repetition of the enzymic experiments with groups of single fibers used as enzyme did not result in complete inhibition of splitting; the experiment, therefore, is inconclusive.

I wish to comment on the potassium iodide and mercuric iodide results. It is very evident that at the end of contraction with ATP, even if one pulls on the fiber with great care, it will break when it is stretched, but after contraction with iodide the fibers are converted into a rubber-like substance.

Such vulcanization, we believe, is a secondary process that arises because the mercury denatures the protein. The initial contraction process we do not interpret as due to denaturation.

GERGELY²: Perhaps it is worth throwing this whole problem into still sharper focus because of the many possible misunderstandings.

As I understand Dr. Morales' theory, it does involve splitting of ATP somewhere in one complete contraction-relaxation cycle, whereas some more recent publications seem rather to suggest that there could be a full cycle without splitting of ATP at all. These experiments of Dr. Bowen's could then not be taken to support, on their own evidence, the view that ATP plays no primary role at all in the full cycle. They might have a bearing on whether the splitting of ATP occurs in the contraction or in the relaxation phase, but I don't think Dr. Bowen wants his results to be interpreted in terms of no ATP participation in the complete cycle.

As to the experiments themselves, I should like to bring up two points here that I am a little worried about, and perhaps Dr. Bowen can throw some light on them. One is whether we can consider shortening as a true reflection of the ability of muscle to do work or should we rather measure tension. In other words, if we want to compare the amount of energy released or utilized as reflected by the splitting of ATP, with mechanical work production, can we use shortening without a load as a sufficient criterion?

The other point is the question of the effect of magnesium on the ATPase activity. If one works at low ionic strength, up to 0.1—at least this has been my experience and perhaps others here can comment on this—magnesium ions do stimulate the splitting of ATP by actomyosin. Under these conditions, actomyosin exists also in the presence of ATP whereas at higher ionic strength ATP causes dissociation into actin and myosin. Some of Dr. Bowen's experiments were

² J. Gergely, Massachusetts General Hospital, Boston

done at an ionic strength as high as 0.3, where only myosin would be found. Now it is known that the splitting of ATP by myosin is inhibited by magnesium.

Although the threads may persist even at a higher ionic strength once they have been formed, in the ATPase experiment they were presumably homogenized. In such homogenized threads, perhaps, you don't have actomyosin in the presence of ATP but only myosin, and you would expect an inhibition of myosin ATPase, whereas in studying contraction you were dealing with actomyosin.

BOWEN: I am going to start with the last question, which is concerned with the relation of ionic strength to the dissociation of actomyosin into actin and myosin. I don't think we are dealing with such a dissociation in the experiments of figure 4, where the ions added to the reaction mixtures, excluding the 0.001 *M* ATP, total 0.1 μ without MgCl_2 and 0.1015 μ with 0.0005 *M* MgCl_2 at which concentration ATPase is drastically reduced. In figure 5 the addition of 0.0001 *M* MgCl_2 to 0.3 *M* KCl should not increase the dissociation caused by the 0.3 μ of the KCl unless MgCl_2 has some specific effect. The inhibitions that start at 0.01 *M* MgCl_2 could very well be due to dissociation into myosin A, which is a less-active enzyme.

GERGELY: That is just the point. I wonder if somehow, under these conditions, the system did dissociate, because according to my experience, if the ionic strength is not more than 0.1 you should get a magnesium stimulation of the splitting of ATP by actomyosin.

BOWEN: I interpret the decreases of ATPase activity caused by concentrations of MgCl_2 of the magnitude of 10^{-4} *M* as specific effects of magnesium in the presence of KCl. If KCl is omitted from these reaction mixtures, 0.0001 *M* MgCl_2 enhances ATPase activity greatly, but the addition of 0.08 *M* KCl, or more, results in inhibition.

The other question was whether the rates of shortening can be used as criteria of energy utilization and the development of tension. I am not in a position to give a complete

answer, but I have proceeded, perhaps naively, on the assumption that the large rates of shortening are correlated with equally large rates of energy utilization. Work is done in shortenings such as I have described even though it is small; however, a final answer will have to await future experimentation, which is planned.

HAYASHI³: So many things have been discussed here that I would like to center attention on just one point. It is well known that the precipitated type of actomyosin thread cannot exert a tension. This is because it is a precipitated gel, in which the micelles are relatively unoriented and unorganized, and the thread does not have structural continuity; that is, there is a lack of intermolecular binding of the component molecules. In such a system the isodimensional change induced by ATP is a complex process involving lateral association of the micelles, a consequent syneresis, and possibly a dimensional change of the micelles themselves. This type of shrinkage, therefore, seems to involve more than the anisodimensional shortening that one might more properly call a contraction.

Dr. Bowen has shown that this shrinkage with ATP does not behave in parallel fashion to the ATPase activity of this system; i.e., whereas ATPase activity declines with increasing KCl concentration, the extent of the shrinkage does not. From this the conclusion is drawn that ATPase activity and contraction are not related, with its obvious inference for muscular contraction.

I raise the question whether this conclusion is justified, on the basis of some doubt as to whether this isodimensional shrinkage should properly be called a contraction. A truer measure of contraction, it seems to me, is the development of tension, or shortening with a load. Fibers of myosin B can be formed, which, by virtue of a greater orientation and condensation of the component molecules, will develop considerable tension or shorten and lift considerable loads upon

³ Teru Hayashi, Columbia University.

the application of ATP. In studies of the dependence of this behavior on the KCl concentration it is found that the development of tension, and the extent of shortening, decreases with increasing KCl concentration. Thus the results lead to the opposite conclusion from Dr. Bowen's; i.e., that the "contraction" and the ATPase activity demonstrated by Dr. Bowen go hand in hand.

It seems clear, therefore, that any conclusions as to the mechanism of muscle contraction based on the behavior of models depend on what sort of *in vitro* change most closely resembles muscular contraction.

OVERBEEK⁴: Of course, I cannot help being struck by the fact that both are ions with a high valency. So I come back to a certain discussion between Dr. Kirkwood and Dr. Flory about the absorption of a neutral molecule or the introduction of extra charge into the contractile system being the important thing.

Just in order to be able to speculate further on the mechanism, I would very much like to know whether the isoelectric point of the contractile system of myosin under the physiological circumstances is known. Do we deal with a positive or with a negative fiber and does the ATP, therefore, increase or decrease the charge?

BOWEN: Dr. Overbeek poses a challenging question and makes it even more so by injecting "physiological circumstances." The isoelectric point of actomyosin solution is elusive in that it is not sharply defined and varies with the KCl concentration (Sarkar, '50); and Sarkar's values were not gathered under "physiological circumstances."

BROWN⁵: In reply to Dr. Overbeek's question, I am not an expert on this but I recall a paper by Dr. Churney, if I remember correctly, in which he showed that pretreatment of muscles would completely change the stimulatory effect of different ions. In other words, if muscle is soaked in acetic

⁴ J. T. G. Overbeek, University of Utrecht.

⁵ J. R. C. Brown, University of Maryland.

acid then put in a base, it will contract; or if it is soaked in a base and then in acetic acid it will contract, and vice versa, which may be of some interest to you.

MORALES⁶: In order to spare Dr. Bowen the misunderstanding with which my similar remarks have at times met, may I emphasize that no one seriously questions, nor do Dr. Bowen's observations imperil, the idea that ATP is split in the normal *over-all* ("complete") contractile cycle. The point at issue is whether the shortening event can temporally precede — and therefore be disengaged from — the step in which orthophosphate is actually split off. Dr. Bowen's several observations *do* bear on this issue, and, in my opinion, they do in fact show that disengagement is possible. This being the case, the observations can be construed to support the idea that the dimensional change is not coupled with ATP splitting, but with the events that *precede* hydrolysis and that are collectively (and perhaps somewhat loosely) called the "binding" of ATP. It is this latter alternative that has been proposed in several publications by my colleagues and me (e.g., Morales *et al.*, '55; Morales and Botts, '56). Now, we have gone further, and have suggested that an electrostatic interaction — in principle similar to that originally envisaged by Riseman and Kirkwood ('48) — is a crucial part of the "binding" process. However, without this additional detail, our "binding" formulation includes several other theories, for example, that outlined in this symposium by Professor Flory, and the very similar hypothesis set forth some time ago by Pryor ('50). Professor Overbeek's query about the isoelectric point of the contractile protein is, of course, very cogent to our ideas. The experimental findings are not so clear as they might be because contraction is best observed in precipitated macroscopic systems wherein classical electrophoresis is difficult to observe. In our opinion, the simplest contraction is brought about because tetra-anionic ATP is adsorbed on *positively* charged Mg^{++} -myosinate. This idea occurred to us originally (Morales and Botts, '52) because

⁶ Manuel F. Morales, Naval Medical Research Institute.

we knew from Professor Szent-Györgyi's early work that Mg^{++} was an essential ingredient of the system, and because it has been repeatedly shown (see for instance, Hamoir, '53) that Mg^{++} adsorption can induce a positive charge on myosin even at pH 's as high as 9.0.

Since Dr. Andrew Szent-Györgyi's and Dr. Hayashi's remarks seem directed more at our hypothesis than at Dr. Bowen's presentation, it is perhaps in order for me to acknowledge them. Apparently they feel that the measurement of shortening rate is somehow not so respectable as that of tension. It is very true that such factors as viscosity could complicate the former and not the latter. It is for this reason that Dr. Bowen and I went to some trouble to show that viscosity was *not* complicating Bowen's observation that Ca^{++} decelerates shortening but accelerates ATPase and that Mg^{++} accelerates shortening and decelerates ATPase. Reciprocally, let me point out a rather obvious pitfall in the measurement of tension, viz., that the macroscopic tension will reflect unitary events only if lateral integrity is preserved. When the system is very fragile to shredding, it may not sustain a comparatively high external tension, but may manage to hold together if it is shortening at very low tension, e.g., its own weight or inertia. But it is fallacious to infer from this behavior that tensions are not being developed at the *microscopic* level; indeed to do so would simply not make physical sense. Once before, we had a classical instance of this mistake. Until Weber ('50) succeeded in producing cross-linked, tension-bearing threads by controlled water withdrawal, people used to say that the myosin thread was a spurious model of contraction, for although it shortened in ATP solution when unweighted, it lengthened when weighted. The unweighted shortening was disdainfully attributed to syneresis (as if the forces of such retraction were somehow not acceptable physical forces!). Now if with increasing KCl concentration Dr. Bowen observes increased shortening rate, and Dr. Hayashi observes decreased tension, I suggest that

what is happening is that at the same time that KCl is *increasing* the tension in the microscopic contractile elements (myosin particles) it is also loosening their lateral adhesions, and that the latter effect is dominating the behavior of the macroscopic tension. I suggest this with some confidence because, as is well known, it is precisely over the KCl range in question that myosin commences to solubilize. It seems very likely that Dr. Andrew Szent-Györgyi's objection can be similarly met. If psoas fibers shorten markedly in iodide or thiocyanate, but fail to develop tension, I suggest that what is happening is that these reagents *are* shortening the microscopic elements but perhaps even more, they are loosening the lateral integrity of the fiber, as indeed they are known to do in other fibrous proteins. The Laki-Bowen observations on the marked shortening by I^- and SCN^- are not more dismissible than the early observations on "ATP-ized" myosin threads. Dr. Andrew Szent-Györgyi may also have forgotten that threads shortened in iodide, then shortened correspondingly less in ATP.

The observation and argument having to do with the enhanced contraction brought about by adding the creatine transphosphorylase system to the myosin-ATP system, was originally set forth by Perry ('55). We have felt that in its original form the experiment lacked the control, myosin system + Mg + ATP + creatine phosphate. However, elsewhere in this symposium Dr. Gergely has suggested what is probably the correct explanation for Perry's observation; namely, that at very low concentrations of ATP, the local ATP concentration is much more effectively sustained in the steady state by a "feeder" system than by ATP addition from the outside. What I find puzzling now is not so much the experiment, but that it should somehow be taken to discriminate between the "binding" and "splitting" hypotheses, since as has been amply acknowledged by almost everyone, the rate of ATP splitting is proportional to the fraction of sites to which ATP is bound, and there is nothing in this experiment to show that the proportionality is disturbed, so

no discrimination seems possible. Some authors have implied that perhaps ATP generated by the "feeder" is somehow more effective than that externally added, but on physical grounds this proposition is quite unacceptable. It is very possibly true that here, as in "relaxation" phenomena, the transphosphorylase molecule interacts in some unknown way with the myosin, but that such a possibility bears on the question at hand is not yet evident.

As to the final point raised by Dr. Andrew Szent-Györgyi, let me say at the outset that why ADP should fail to cause contraction is certainly obscure to me. At the same time, I do not think we are in the paradoxical situation of knowing that ADP binds strongly to the same sites as does ATP. I have tried in vain to show ADP binding by equilibrium dialysis; and I suppose that many others have failed likewise or else they would have published so significant an experiment. It is true that Blum ('55), and more particularly Green and Mommaerts ('54), have reported seemingly competitive inhibition of ATPase by ADP. This of course is not a rigorous indication that the two substances bind to the *same* site nor that ADP would bind in the absence of ATP (see Botts and Morales, '53, and a forthcoming publication by Botts on the myosin-EDTA-ATP system), and it is not clear that a non-dispersed myosin system would have contracted in those milieux, but it must be admitted that it is a significant problem for us to study and overcome. Our view of contraction requires that there be forces in addition to the purely electrostatic ones binding the ATP molecule to the myosin site; presumably, maximum interaction is achieved only when both ring and triphosphate moieties are on the same framework.

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THE KINEMATICS OF RETRACTION IN CONTRACTING STRIATED MUSCLE¹

FRANCIS D. CARLSON

*Department of Biophysics, The Johns Hopkins University,
Baltimore, Maryland*

TWO FIGURES

In discussing muscular contraction I would like to confine my remarks to the problem of characterizing the contractile process in terms of appropriate physical variables. The problem is important for two reasons. First, a physical characterization of the contractile process at the phenomenological level will undoubtedly help in suggesting molecular mechanisms for the process. And second, contractile mechanisms derived from structural and biochemical studies can be rigorously tested only by comparison of the mechanical behavior predicted by them with that of actual muscle. Even if the necessary structural data were available for quantitative calculations based on the proposed physical theories of contraction, we could not rigorously check these theories at the present time because of our incomplete picture of the contractile phenomenon.

Several approaches to the physical characterization of the mechanical properties of a system are possible. All have been used on muscle. Thermodynamic methods, such as load extension, thermal expansion, and stress isometric studies have been utilized. Their interpretation is hampered, however, by the failure of muscle to satisfy the conditions of equilibrium, reversibility, and freedom from chemical reactions. Mechanical impedance determinations have been made

¹The research reported here was supported by a grant from the USPHS, RG-3723.

on muscle, particularly on single fibers, but the interpretation is not unequivocal, chiefly because of the nonlinear behavior of muscle. The third approach is through a study of the kinematics of muscular contraction, under various constraints, and the deduction of the mechanical properties of the system from its kinematic behavior. In principle, the method is similar to the mechanical impedance method, but we have chosen to differentiate between the two because the assumptions of linearity and superposition ordinarily made in the interpretation of impedance studies are not required in the kinematic method. Such studies have long been used to characterize the contracted state of muscle, and indeed Hill's well-known load-velocity relation ('38) is based on studies of this type.

As the name suggests, kinematic studies are concerned with the motions of the muscle during retraction. The interest is in the motion of each point along the length of the muscle as well as the motion of the free end, and in how these motions depend on the initial length of the muscle, the load, and other parameters. From such data one hopes to formulate empirical laws that characterize the motions and then relate the constants appearing in such laws to structural and chemical changes occurring within the muscle during contraction. The characterization of muscular contraction presented here is based on the results of our kinematic studies, the details of which will be published elsewhere.

First, the motion of each point along the length of the muscle during shortening or retraction will be considered. When constrained with loads less than the maximum tension that the muscle can develop, a stimulated muscle will shorten from its initial rest length to a new equilibrium length whose value is determined by the value of the load. The process can be likened to the retraction of a visco-elastic body that has been stretched and then released. Figure 1 shows the trajectories of points on such a body for three different types of retraction processes. In figure 1A, inertial forces dominate over viscous

forces and a wave of acceleration travels down the body with a velocity determined by the Young's modulus and the density of the material. Each point in the material remains stationary until the wave reaches it and then begins to move with a constant velocity. The diagram shows the motions only up to the time the wave of acceleration reaches the fixed end. After this, the motion becomes complicated by a reflected wave. In figure 1B, viscous forces are dominant over the inertial forces and retraction begins everywhere along the muscle at the same time, proceeding monotonically to the new equilibrium length. In 1C, the viscous forces are domi-

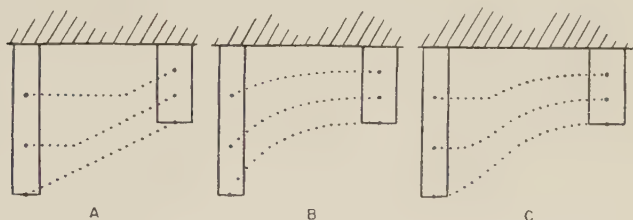


Fig. 1 Retraction trajectories for different types of retraction processes: A, inertial forces dominant; B, frictional forces dominant; C, release mechanism with frictional forces dominant.

inant but the material is released gradually, proceeding from the free end to the fixed end, and not instantaneously as in A and B. In this instance, retraction begins at a point only when the release mechanism has acted there and then it proceeds as in B. The process drawn schematically in C is similar to that occurring in normal muscle when electrically excited over a small region near the free end. The propagated action potential proceeds up the muscle, leaving the contractile process activated in its wake. Clearly, the motion of the free end of a muscle so excited is determined by both the velocity of the propagated impulse and the retraction characteristics of the contractile material. The study and the interpretation of the kinematics of retraction would be simplified if the com-

plications introduced by the propagated release mechanism were removed.

We have found, in agreement with Bethe and Happel ('23), that by using a strong electrical stimulus between the two ends of the muscle we could initiate contraction virtually simultaneously at all points along the muscle, and a situation such as is diagrammed in figure 1B obtains. The motion of points on the muscle was determined by high-speed photography of retracting isotonicallly loaded muscle, which had been marked at intervals of 3-4 mm along its length with small pieces of thread. For strong end-to-end electrical stimulation, the records obtained are similar to figure 1B. For weak electrical excitation, however, the propagated response obtains and the records are similar to figure 1C.

When the muscle is so excited that all points begin to shorten simultaneously (fig. 1B), the time of retraction for each point on the muscle is the same. The amplitude at a given point is reduced in proportion to the ratio of the distance of the point from the fixed end to the total length of the muscle. The evidence for this is obtained from the photographs of retracting muscle by tests for statistical significance of the hypothesis that the extension ratio-time curve for each segment of the muscle is the same as that for the entire muscle. If this were so, then at any time during retraction, the difference between the extension ratio for the whole muscle and the extension ratio for any single segment of the muscle should be zero within the limits of experimental error. The time average of this difference was obtained on each of eight different segments along the muscle and the significance of its departure from zero was tested by Student's test. The *P* values obtained ranged from 0.2 to 0.45, indicating that the retraction of the segments does not differ significantly from that of the total muscle. Since the retraction is homogeneous along the length of the muscle, observation of the motion at the free end is sufficient; the motion at other points is the same as that of the free end but diminished in amplitude

in the manner stated. For this type of retraction, it follows that a single sarcomere 3μ long has the same half-time of shortening as does the entire muscle. Accordingly, the 3μ sarcomeres in a muscle fiber 3 cm long (10^4 sarcomeres) fired simultaneously and each shortening at a speed of 20 μ /second, would produce a 20 cm/second shortening velocity of the free end of the muscle.

It should be pointed out that the retarded elastic behavior shown in figure 1B is obtained only for an after-loaded contraction; that is, a contraction in which the load is supported initially by a stop, and shortening begins only when the tension in the muscle becomes equal to the value of the load. If a muscle is allowed to develop its full isometric tension and then released suddenly, there is an initial rapid, pure elastic-like, shortening and then the retarded elastic behavior described. The kinematic behavioral equivalent of muscle therefore requires an elastic element in series with a retarded elastic element, or its equivalent.

Kinematic studies on after-loaded contractions show that the retarded elastic element is nonlinear. Evidence for this is shown in figure 2, which is a plot of the instantaneous velocity against the instantaneous displacement of the free end of the muscle for various initial lengths and loads (Carlson, '54). If the retarded elastic element were linear, these plots would show a sudden change in velocity and then a linear dependence of velocity on displacement. An analysis of displacement velocity plots such as these shows that the nonlinear retraction behavior is consistent with a model comprised of a nonlinear elastic element in parallel with a nonlinear viscous element. By a nonlinear elastic element and a nonlinear viscous element we mean mechanical elements whose stresses are nonlinear functions of the strain only and rate of strain only, respectively.

It is now apparent that a complete physical characterization of muscular contraction requires not only data on the tension-length characteristics of muscle but also information on its retarded elastic-like behavior. Linear retarded elastic be-

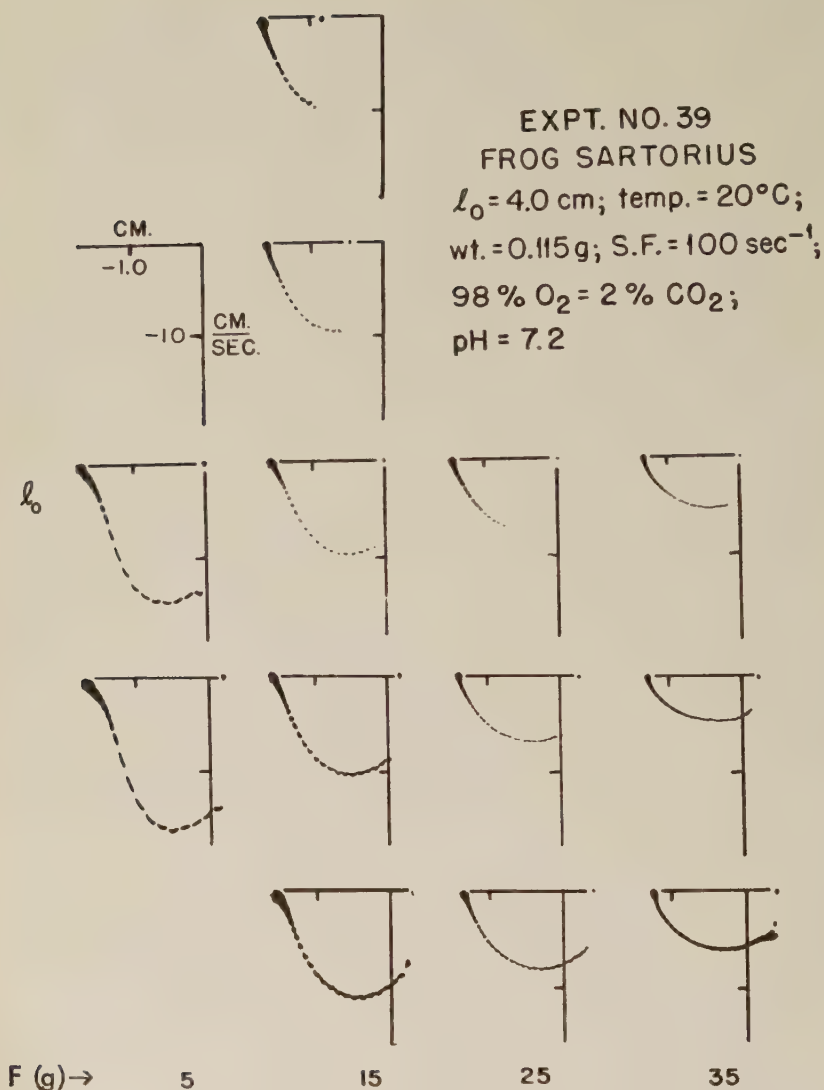


Fig. 2 Displacement-velocity plots for retracting muscle, tetanically stimulated. Columns correspond to different values of isotonic load, rows to different initial lengths.

havior in isotropic polymer structures can be characterized by a retardation time or distribution of retardation times (Alfrey, '48). When a single retardation time is involved, its value is given by the ratio of the coefficient of internal friction to the Young's modulus for the material. Furthermore, the slope of the velocity-displacement curve (a line) is equal to the retardation time. A formalism does not exist that permits the treatment of nonlinear retarded elastic elements in terms of a retardation time or spectrum of retardation times. Under certain conditions, however, we may sometimes obtain useful information about visco-elastic properties of nonlinear systems by considering their behavior in the limit of zero strain. This, we believe, can be done with muscle.

The conditions satisfied by the retraction behavior of tetanically stimulated muscle that enable us to define and evaluate a retardation time for its retraction behavior in the limit of zero strain are as follows:

1. The retraction is uniform along the length of the muscle consistent with the behavior of an isotropic homogeneous visco-elastic material whose mechanical behavior is dominated by internal frictional forces.

2. To a first approximation, the elastic forces and viscous forces are independent since the total forces developed by the muscle can be represented as the sum of two functions, one a function of the displacement only (elastic forces), and one a function of velocity only (frictional forces).

3. In the limit of zero strain, the length-tension curve of muscle becomes very nearly linear, and a Young's modulus can be estimated from the length-tension curve; the cross-sectional area of the muscle is estimated by division of the weight of the muscle by its length.

4. As the retraction nears completion, the displacement-velocity plots (figure 2) approximate straight lines, and retardation times can be estimated from their slopes, which are in fair agreement with those estimated from the "time constants" of the retraction curve for the free end of the muscle.

On the basis of these considerations, the Young's modulus and retardation times were determined from the retraction data on frog sartorius muscle. Table 1 summarizes the values for these quantities and includes the calculated coefficient of internal friction. The Young's modulus in table 1 is computed both for the cross section of the entire muscle and for the estimated cross section of the contractile proteins (myofibrils) present in the muscle. The accuracy of values given is not known, but it is expected that they are at least of the correct order of magnitude.

Some knowledge, or an assumption, about the general nature of the contractile process is needed for the interpreta-

TABLE 1
*Empirical constants of the equivalent retarded elastic model
of frog sartorius muscle*

CROSS SECTION REFERRED TO	YOUNG'S MODULUS	RETARDATION TIME	COEFFICIENT OF INTERNAL FRICTION
	$E \times 10^{-6} \text{ dyn/cm}^2$	$f/E \text{ sec}$	$f \times 10^{-6} \text{ poises}$
Whole muscle	5	0.037	0.2
Myofibrils	60	0.037	2

tion of the mechanical constants of muscle shown in table 1, in terms of the molecular mechanism of contraction. That is, if we accept the currently popular hypothesis, which is by no means proved, that on excitation resting muscle is converted into a new visco-elastic body having mechanical properties and equilibrium length different from resting muscle, the constants given can be regarded as characteristic of the visco-elastic material of the contracted muscle. Accordingly, these constants can be interpreted in terms of modern theories of the visco-elastic behavior of high-polymer systems. On this basis the Young's modulus given for muscle in table 1 is consistent with the idea that the elastic forces of muscle are of configurational entropic origin. The nonlinear length-tension relation, the rapid elastic behavior, the retarded elasticity, and the independence of the elastic and viscous forces are all

consistent with the known mechanical behavior of polymer structures. The data available on the retardation time are altogether too meager to warrant a detailed interpretation. They do, however, invite a few comments if for no other purpose than to indicate the type of information that might be obtained from future studies. First, if true, it is interesting that the retraction can be characterized by a single retardation time or at least a very narrow spectrum of retardation times. Most high-polymer structures, depending on the temperature, show a rather broad spectrum of retardation times, a consequence of a distribution of mechanisms associated with configurational changes in the high-polymer molecules that act simultaneously to produce the visco-elastic spectrum. A single retardation time is hardly to be expected if configurational changes are dominant in the retarded elastic behavior. Its occurrence suggests, therefore, that some other mechanism, possibly a chemical reaction, a phase change, or dynamic process that can be characterized by a single time constant, is primarily responsible for the retarded elasticity. The magnitude of the internal frictional coefficient (10^6 poises) also deserves comment. Such high-frictional coefficients are found in materials such as rubber, which is combined with finely divided carbon black. It is not immediately apparent how those models of muscle that depict the contractile system as a highly swollen, cross-linked, amorphous gel whose elastic forces are of an entropic origin can have such a high value for the internal frictional coefficient. We expect that studies on the effect of temperature on the retardation time, now in progress, will provide information on the type of process responsible for the retarded elastic behavior.

As mentioned, the interpretation of the retarded elastic behavior of muscle in terms of an elastic modulus and coefficient of internal friction is based on the hypothesis that stimulation converts resting muscle into a new visco-elastic material with characteristic mechanical constants and equilibrium length. Although this hypothesis is plausible and

useful in that it enables one to interpret experimental results in terms of modern physical theories of visco-elastic behavior, it remains unconfirmed. The contractile process might well be the result of an entirely different type of mechanism. There is no a priori reason why the nonlinear mechanical behavior of muscle could not be the consequence of complex series of processes at the submicroscopic level, a machine so to speak, rather than the exclusive result of the configurational changes occurring in a cross-linked polymer structure created by chemical reactions activated by stimulation. In addition to the nonlinear mechanical behavior, which prompts speculation on possible dynamic mechanisms, contraction is essentially transitory, as evidenced by the twitch itself, and needs repetitive stimulation for sustained activity (tetanus). One mechanism of this type, offered here as a conjecture, would require that each sarcomere of the muscle fiber contain two sets of structures that would be bounded by sliding surfaces, presumably small, such as the surfaces separating small crystallites in polycrystalline materials. One set of these structures, at least, would be tightly coupled to the external covering of the muscle fiber (sarcolemma), which would enable it to transmit tensile forces to the tendons. The interpenetrating, close-packed, hexagonal structure proposed by Hanson and Huxley ('55) for muscle is precisely such a structure. There would then be superimposed on the quasi-elastic force field that holds the molecules of one such structure in their equilibrium positions the periodic force field of the structure present on the other side of the sliding surface, and vice versa. The equilibrium positions of the molecules in the one structure are determined by its own force field and also by the superimposed force field, and hence by the relative position of the other structure. It is then supposed that, upon stimulation, an active process is initiated that produces a transient change in the spatial distribution of one or the other of these force fields. Possibly this change is the result of a chemically induced deformation of the structure, or of a change in the

charge distribution also chemically induced. In any case, the change in the spatial distribution of the superimposed force field would give rise to a new set of equilibrium positions for the molecules in the other structure, with a resultant reactive force in the direction of the fiber axis. If restrained, such a set of structures would show a transient tensile force; if not, they would show a transient length change. A structural model of this general type has been worked out in detail by Ludwig Prandtl to explain the hysteresis and relaxation effects shown by polycrystalline materials, and the possible significance of such a model to phenomenological theories of relaxation and viscosity has been discussed by Burgers ('39). Because of the transitory nature of the process producing the change in the force field, the tension developed, or the retraction, would rise to a maximum and decay to zero with time constants determined by both the activating process and the relaxation time of the interacting structures. Repetitive activation would bring about a net average change in the spatial distribution of the superimposed force field, with a resultant sustained tension or shortening.

At the present time, a decision does not seem possible between "elastic body" theories, "dynamic process" theories, or any other type of plausible theory, on the basis of existing structural, chemical, or mechanical data. It would appear that what is needed is a more accurate and complete understanding of what structural elements are actually deformed during shortening or lengthening and exactly how they are deformed, together with a quantitative characterization of the associated macroscopic mechanical events in terms of well-defined physical variables. These data would enable us to estimate the magnitude and distribution, at the submicroscopic level, of the forces responsible for contraction. A comparison of the characteristics of these forces with the physical and chemical properties of the contractile proteins in solution and in aggregated states, in the presence and absence of adenosinetriphosphate and other compounds, should shed light on the actual mechanism of muscular contraction.

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FIBRILS AND FILAMENTS OF MUSCLE PROTEINS AS EXAMPLES OF MOLECULAR COMPLEXES

RICHARD S. BEAR

*The Department of Biology, Massachusetts Institute of
Technology, Cambridge*

ONE FIGURE

The application of X-ray diffraction methods to the elucidation of tissue ultrastructure has been largely concerned with derivation of the physical configuration of the macromolecular substances present. Considerable progress has now been made in determination of the ways in which atoms or residues are arranged along the molecular chains of fibrous systems. Successes in arriving at helical configurations for polypeptide (Pauling and Corey, '53b) and polynucleotide (Wilkins *et al.*, '53; Watson and Crick, '53; Franklin and Gosling, '53) structures have resulted in widespread interest in molecular coils. Less well known are similar structures under consideration for some time for the iodine and other complexes of amylose (Hanes, '37; Freudenberg *et al.*, '39; Bear, '42; Rundle and French, '43; Senti and Witnauer, '52) and for certain forms of alginate, pectate, and cellulose (Palmer and Hartzog, '45). Three-chain, coiled-coil structures for collagen molecules have now been supported by four, more or less independent, lines of evidence (Rich and Crick, '55; Cowan *et al.*, '55; Ramachandran, '56; Bear, '56).

Important as these structures may be, they furnish in reality only elementary beginnings for an understanding of the intra- and extracellular fibrous forms encountered in biological organisms. Although several cases could be discussed by way of illustration, for present purposes the proposed structures of

paramyosin fibrils (Bear and Selby, '56) and actin filaments (Selby and Bear, '56) will serve as examples.

These two fibrous elements of muscles have been amenable to analysis of their large-scale order because they yield reasonably detailed and interpretable small-angle X-ray diffraction. In a limited sense, not yet fully definable, the paramyosin of molluscan muscle may probably be taken to represent the α type of fibrous protein occurring in muscles of more familiar (vertebrate) animals under the name myosin but usually presenting greater difficulty for large-scale structure determination. Actin filaments are more constantly constructed in a wide variety of muscle types. Together, the paramyosin and actin structures furnish prototypes of the two main kinds of fibrous protein constituting the bulk of the contractile apparatus of any muscle.

Figure 1 illustrates diagrammatically the structures derived for these two elements, presented in more graphic fashion than was given in the original crystallographic analysis. In paramyosin, the manner of distribution of scattered X-ray intensity, to either side of the small-angle pattern meridian, indicated that the fibrils contain "rods" of diameter about 100 Å lying with axes along or at a small angle to the fibril axis. One way that these could run through the fibril is shown in figure 1A. Interruptions are spaced along each rod to indicate the size of the structural periodicity they must also possess along the rod axis.

The small-angle diffraction of the actin component (in intact molluscan muscle) resembled that of paramyosin in certain respects, so that the analysis proceeded similarly. The sizes of the comparable features were, however, appreciably smaller for actin, as figure 1B shows. Thus the actin rods are thinner (25–50 Å) than the paramyosin rods; indeed, the entire actin filament is probably thinner than each paramyosin rod.

There may be another important difference between the paramyosin and actin structures. Figure 1A represents only

a ribbon-like layer of the total molluscan fibril, as judged from electron-optical studies of Hall *et al.* ('45). Over the plane of the layer surface the periodicity of structure is net-like in a fashion indicated in figure 1A after dictates of the observed relative positions of the small-angle X-ray reflections. The actin structure may also be net-like, but technical

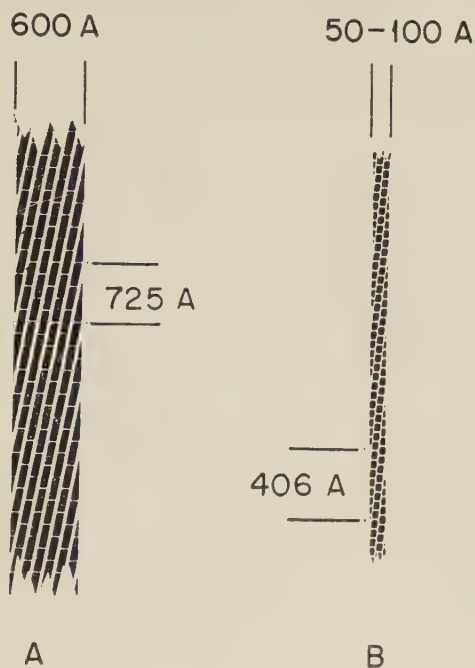


Fig. 1 Diagrammatic representation of the large-scale structure of A, the paramyosin fibril, and B, the actin filament, as found in intact molluscan (*Venus*) muscle. The actin model is at twice the magnification of the paramyosin structure.

difficulties prevent ready distinction of nets and large-scale helical structures at small diffraction angles. Electron micrographs of isolated actin filaments (Rozsa *et al.*, '49) suggest that actin filaments are helical. In that event, figure 1B should be rolled so that vertical edges coincide to form a filament composed of two rods twisted about each other.

Little further detail is known of the manner in which paramyosin rods and actin filaments may be built into or

interact within molluscan muscles. The myosin of the myofibrils of striated muscles is believed to be located in A (anisotropic) bands, where the thicker filaments, of about the same diameter as that of paramyosin rods, are said to be in large part myosin (Huxley, '53; Hanson and Huxley, '53). The thinner actin filaments predominate at I (isotropic) bands and probably extend along most of the sarcomere length, though details of the relations of these two components are still incompletely agreed upon (see Hodge, '55; Spiro, '56). Leaving for the future these higher-level problems of the organization of myofibrils one can still illustrate the complexity of the molecular aggregates involved by attention to the paramyosin rod and the actin filament as separate entities.

Possibly the most fundamental fact known about the molecular chains of paramyosin is that, like all other members of the α subclass of the keratin-myosin-epidermin-fibrin family of fibrous proteins (see Astbury, '47), the configuration of the main peptide chain is approximately that of the α -helix. The ideal α -helix as originally proposed by Pauling and Corey ('51) has been most nearly realized in certain synthetic polypeptides (Yakel *et al.*, '52; Cochran *et al.*, '52; Bamford *et al.*, '53). Native α proteins have, however, more heterogeneous populations of side chains that result in several individual helices (two to seven) twisting about a common axis to form cables of coiled coils (Pauling and Corey, '53a; Crick, '53). Since individual α -helices have diameters of 10–12 Å, the cables are at most 30–36 Å thick. A still thicker (ca. 100 Å) paramyosin rod would be an aggregate of cables, or perhaps even a supercable formed by twisting the cables about the rod axis.

The periodic structure along the paramyosin rod would be a function of the various twists imposed on the constituent α -helices and/or the way in which individual molecules are arranged along the rod axis. Hodge ('52) described dispersion in acid solutions of long, thin particles approximating single

helix to cable thicknesses and 1000–1500 Å long. Corresponding large particles of myosin can, by short tryptic digestion, be broken further to meromyosins (Szent-Györgyi, '53), one of which (light meromyosin) in turn has been disaggregated in urea to protomyosins of particle weight ca. 5000, which are physically, though not chemically, fairly homogeneous (Szent-Györgyi and Borbiero, '56). It is apparent that considerable molecular complexity exists in these α -type muscle proteins, in addition to the complications of rod or filament structure.

Practically nothing is known about the molecular-chain configuration of actin. It is not obviously that of the α -helix, since the rather poorly developed wide-angle X-ray diagram of reconstituted films and fibers is not an α -type pattern (Astbury and Spark, '47; Cohen and Hanson, '56). The reconstituted fibers otherwise show excellent small- to moderate-angle diffraction similar to that part of whole muscle diffraction used to derive the structure of figure 1B. Indeed, it was the results just cited from isolated actin that finally proved that the filamentous component of whole muscle described in figure 1B is actually rich in actin.

The molecular weight of actin is believed to be about 57,000 (see Mommaerts, '52). This is very roughly the size of one of the block-like segments, 55 Å long, shown in the model of figure 1B to form the individual actin rods by aggregation in series. On the other hand, if the more asymmetric molecular shape (290 by 24 Å) proposed by Tsao ('53) is correct, then the actin rod's cross section may contain up to four molecules, each of which would extend over about five periodic segments of the rod.

These examples show that in addition to knowledge about chain configuration, better information about weight and shape of molecules is indispensable. They also disclose that details of the ways in which the molecules aggregate are equally necessary, if the complexes that form the colloidal elements of muscle fibrils are to be understood. Required will be methods of analysis that can isolate or otherwise

examine, as well as molecules, the significant aggregates at cable and supercable or rod levels of organization. One may be hopeful that at least the kinds of organization to be scrutinized are now known from the molecular to the myofibrillar level.

ACKNOWLEDGMENTS

The author is pleased to acknowledge helpful discussions on the components of muscle with Drs. Carolyn Cohen and Andrew G. Szent-Györgyi, with whom a survey of X-ray diffraction and optical properties of isolated muscle proteins is now in progress. The portion of this program currently undertaken in this laboratory constitutes a part of investigations supported by the National Science Foundation.

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REMARKS ON PROTEINS: SUMMARIZING STATEMENTS

ALBERT SZENT-GYÖRGYI

*Institute for Muscle Research at the Marine Biological Laboratory,
Woods Hole, Massachusetts*

I want to make my remarks on proteins led by experimental work that is not my own but that of Drs. Gergely, Mihalyi, and Andrew Szent-Györgyi, who just happened to work in my laboratory.

To make myself clear I will have to go back a little in the history of myosin. Ten years ago the myosin molecule looked like a simple thin rod, and we were all happy because in those days, once one gave the relative over-all dimensions of a molecule one had said everything about a protein that had to be said. (Since then, Dr. Laki has cut the myosin molecule in two, showing that the old molecule was probably a dimer of a smaller monomer.) In the earlier days we had no doubt about the nature of contraction. Nobody doubted that it is some sort of folding. Whether the folding was random or occurred at only certain points with straight stretches between did not seem important. There was folding and I, myself, felt sure that it was a regular folding, and ATP, when producing contraction, acted on the points of the folding.

This model has since been complicated very greatly by the work of Gergely, Mihalyi, and Andrew Szent-Györgyi, which showed that the myosin molecule is built of three subunits shunted in series. There are two kinds of such subunits within one molecule. The one kind was called (because of the low sedimentation constant) "L-meromyosin" (L standing for light), and the other was called "H-meromyosin" (H standing for heavy). There are two L's and one H in one molecule. We

do not know their sequence but the most reasonable assumption would be that the H is in the middle.

There is little doubt that these units are really preformed. They are not just artifacts but they are really subunits that are there within the myosin molecule since the reactions and activities of the myosin molecule are shared between the two. It is only the H that combines with ATP and splits it, and we have reason to believe that the L is involved in contraction because its reactions show the same dependence on ionic concentrations as does muscular contraction.

I do not know the meaning of all this but I am sure it has one. The first remark I want to make about proteins is this: if we study a protein and try to find out its structure there is one question we mostly forget to ask and that we always should ask, namely, that about the meaning of the structure found. The essential question to me is not how a protein is built, but why nature has put those atoms together in that very specific order. What was the property nature wanted to achieve by putting that great number of atoms together in that very specific way?

Another remark I want to make about proteins more specifically is in connection with the distribution of ATPase activity. There is now a fairly good agreement that the energy of contraction comes from the ATP molecule and that the utilization of that energy is connected with the splitting of the ATP molecule in one way or another. If the L meromyosin is what contracts and produces work and it is the H that liberates the energy of ATP, then one has to suppose that the energy has to go, somehow, from the H to the L.

Here one is faced with a very basic problem of how energy moves the muscle. This is not a specific question limited to myosin but one of the most general biological problems. This problem is: how does energy drive the living machine? To my mind this is one of the most fundamental biological questions.

One could advance two different theories. Taking muscle as example of the living machine, one could picture the action of ATP as a local point action, producing a local change on the molecule by some classical chemical interaction. This is one possibility. We could say, for instance, that contraction comes about by points here and there, losing or increasing their charge on the myosin molecule. This then could produce some sort of a folding, a doubling-up and herewith shortening. So contraction could be explained, tentatively, by a purely local action of the ATP that would change something only at one single point in a reaction which could be described by classical chemical symbols, by letters and dashes between.

The other logical possibility would be to suppose that the energy is released from the ATP molecule in some active and mobile form capable of moving, and capable of diffusing through a system, and that it goes from the H-meromyosin to the L, producing changes there. At the moment, we cannot decide between the two possibilities outlined because we have too few data. I have spent the last 4 years exploring the second possibility and the more I see of it the more I begin to believe in it. There are observations that speak greatly in its favor. One such observation, for instance, is related to studies of the bacterial flagella (*Bacterium Protei*). Those flagella are 2μ long; in atomic dimensions, this is miles and miles. There are reasons for believing that the energy that moves a flagellum is liberated at its base and starts up a wave of contraction, which then runs along this very long fiber that must work all along to drive the bacterium forward. These flagella are very thin — only 130 Å in diameter. So they can consist of only a simple strand of protein fibers and there can be no tube in the middle to supply ATP or any other chemical energy source. We have to assume that the energy, produced at the base of flagella, moves along this fiber and is dissipated on its way as it does work.

This complicates our problem very much, because if we suppose some mobile form of energy we also have to suppose

some structure within the protein that conducts that energy. About 15 years ago, Dr. Laki and I speculated about this problem and came to the conclusion that proteins may be semiconductors. Since then, Dr. Gergely, with M. G. Evans, worked on this problem and found evidence for continuous energy bands in proteins, though the evidence was not quite conclusive. There are several possible ways to propagate energy through proteins but I do not want to go into them. Suffice it to say that in order to have a mobile form of energy, we would have to have some structure in the protein that can propagate that energy. Here I come back to my first remark because if there is such a structure, let's say, a conduction band, there must also be a very specific atomic configuration, and nature may have put those atoms together in that very specific way to achieve fusion of the energy levels of atoms to a band on a quantum mechanics basis (and nature seems to know a great deal of quantum mechanics).

This was the situation with muscle 15 years ago, but today the picture is still more complex, and this is due to the work of Andrew Szent-Györgyi. A decade or so ago I built a new thermodynamics of muscle, based on the assumption that the contractile matter consisted of very small subunits that acted independently from one another in an all-or-none equilibrium reaction. I was the only living creature in this world who believed in this theory. Andrew believed it half-heartedly. He said: "If there are really units as you suppose, also outside your head, then one should be able to find them."

So Andrew began to tease the myosin molecule and soon he found that under very specific conditions — at a special concentration and temperature — this protein goes to pieces if put in urea. It falls into very small pieces of equal size, of a molecular weight of 5000 g. So if the L-meromyosin has a molecular weight of 100,000 g, then it is built of twenty such subsubunits. The really dramatic feature of this discovery is that these units are held together by secondary forces only. If there ever was a "molecule" in biochemistry,

it is the myosin molecule, because it is there to produce tension and so has to withstand strain. Now it turned out that this molecule is not a molecule at all. It is a conglomeration, a regular heap of very small units held together only by secondary forces.

What does this mean? Let us consider first the mechanism of contraction. It probably means that contraction involves some rearrangement in the relative position of these very small units, "protomyosins." So if the energy of the ATP molecule has to move the myosin it has to do something to a greater number of such units and the forces holding them together. How it can do this we do not know since we do not know what contraction is. We do not have the least idea, and the more we know about muscle the less we understand it. If this goes on in the end we will know everything and understand nothing.

Of course, everybody has his own pet theory of contraction and his pet model, of which there is a great number now on the market. But I am afraid the situation is similar to that of the holy elephant that had ninety-nine names, the real one being the hundredth, known only to the elephant himself.

There are questions that come up in one's mind in connection with this structure of myosin. The characteristic of this molecule is that it is composed of small basic units of molecular weight 5000 g. My experience is that nature works with a few basic principles and not with exceptions. If we find something striking in one place then we usually find a basic law behind it, and later find the same law applied in other places over and over again.

So the experience with myosin might mean that the basic units of protein structure are of the order of magnitude of the protomyosins, but there is no proof for this. One may also think that myosin is an exception, nature having made it from small units because myosin is the only molecule that has to move; if it were built of long filaments, it could not move or change its shape. I am inclined to think the other

way; that is, to suppose that the architecture of myosin represents a basic principle that all proteins are constructed likewise. Myosin is an exception only in so far as its basic units are loosely connected to enable the whole protein to move. Insulin is also built of very similar units of molecular weight 6000 g and Dr. Waugh just told you in his very beautiful lecture how these can join to make a very specific and stable fiber. If this represents a basic blueprint, then protein synthesis is accessible to a new interpretation. The protein synthesis might go then in two steps, the first being the building of these little units and the second the putting of them together. They may get together spontaneously, as Dr. Waugh's insulin fibers, but they may also need an organizer for this act. The one of these functions may be performed by DNA and the other by RNA. Whatever the case may be, these observations on the structure of myosin pose new problems.

Keratin has also been decomposed into smaller units. If one is unable to decompose all other proteins into such small units, this will not take away the possibility that they have the same basic structure, only they may have their "proto-proteins" held together by means of covalent bonds, no mobility being needed.

I would like to sum up my remarks on proteins by saying that we should not be content to ask questions about the structure of proteins but should inquire also into the deeper meaning of the structures found. Owing to its specific function, generation of motion, nature has endowed the myosin with specific qualities that open new ways for its analysis and that may possibly lead to new basic concepts about protein structure and function.

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CURRENTS OF THE VISUAL CORTEX

IN THE CAT

Supp no 2

JOURNAL OF CELLULAR AND COMPARATIVE PHYSIOLOGY

Volume 49, Supplement 2, June 1957

CURRENTS OF THE VISUAL CORTEX
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By
WOLFGANG KÖHLER
AND
DONALD NEIL O'CONNELL

Published by
THE WISTAR INSTITUTE OF ANATOMY AND BIOLOGY
PHILADELPHIA, PA.

CURRENTS OF THE VISUAL CORTEX IN THE CAT¹

WOLFGANG KÖHLER AND DONALD NEIL O'CONNELL

*Department of Psychology, Swarthmore College,
Swarthmore, Pa.*

THIRTY-EIGHT FIGURES

INTRODUCTION

Currents of the auditory cortex have been recorded both in man and in the cat (Köhler and Wegener, '55; Köhler, Neff and Wegener, '55). In the former case, a large distance and media of high impedance intervened between the active tissue and the crucial electrode. Nevertheless, similar curves were obtained in both species. Under the circumstances, it seemed natural to expect that currents of the cat's visual cortex would also closely resemble those of the human striate area. But when, during the study of the cat's auditory currents, some records were taken from the visual cortex of the preparations, the currents established by light differed from human visual currents in one respect: while currents registered from the human scalp over the cortical fovea indicate a surface-positive flow through the source, the visual currents of the cat had the opposite polarity. Their direction was surface-negative just as is that of auditory currents both in man and in the cat.

This finding may be interpreted in two ways. On the one hand, it may lead to doubts as to whether the polarity of visual currents as registered from the intact human head agrees with their polarity at the source. In view of the complicated geometrical and physical relations which obtain between the

¹ This investigation has been supported by a grant from the Carnegie Corporation. It has been carried out in the Pavlovian Laboratory of Johns Hopkins University. To a large extent, the present publication has been made possible by a grant from the Commonwealth Fund.

human striate area and an electrode attached to the scalp over the cortical fovea, such doubts deserve serious consideration. But since they would not arise if the currents of the cat's visual cortex also showed the surface-positive direction, conclusive evidence that the direction of these currents is, in fact, surface-negative must obviously be obtained in the first place.

On the other hand, it might be said that there is no particular reason why currents of the visual cortex should have the same direction in man and in the cat. But, if they have opposite directions, one would expect vision in the cat to differ in some respects from human vision. For it has been shown that currents of sufficient intensity affect cell layers of the brain in one way or another depending upon the direction in which such currents pass through the cells (Gerard and Libet, '40; Goldring and O'Leary, '51). Again, however, considerations of this kind have little weight so long as the surface-negative direction of visual currents in the cat is not yet firmly established. We therefore decided to subject these currents to more systematic tests.

Our experiments were done in the Pavlovian Laboratory of the Medical School at Johns Hopkins University. We are grateful to Doctor Gantt for his kind hospitality. To Doctor Kuffler of the Wilmer Institute and to Doctor Bard, Doctor Rose, Doctor Mountcastle, Doctor Henneman and Doctor Elrunkar of the Department of Physiology we are indebted for much valuable advice and technical help.

TECHNIQUE

The surgery necessary to expose the dura was mostly done under pentothal, only in some instances under nembutal. Generally, the injections were given intraperitoneally. Smaller quantities of the anesthetic were later injected intravenously when movements of the cat began to disturb the recording. On the other hand, application of picrotoxin became necessary in a few instances. Only in some animals were large parts of the skull removed; more frequently, one or several trephine holes

were made over the visual projection area. Within this region, the location of the exposed parts was chosen on the basis of the maps given by Talbot and Marshall ('41). Most records were taken from the region corresponding to the *area centralis* or to slightly higher parts of the retina. In some instances, only one eye was kept open, in others both were used during the tests. When the eyes had to remain open for considerable periods, they were protected by contact lenses or by mineral oil. In most cases, atropin was used. During tests, the cats were restrained by keeping the head fixed in a head holder, and by fastening the legs to an animal board.

We used electrodes of the silver-silver chloride type, inserted in a glass tube (*cf.* Köhler, Held and O'Connell, '52, p. 296). Contact between their core and the dura was established by short wicks moistened with saline. The area of contact had a diameter of about 3 mm. In most cases, the grounded electrode was attached to the animal's neck. Our D. C. amplifier, an instrument of the breaker type, has been described elsewhere (Liston, Quinn, Sargeant and Scott, '46; Köhler, Held and O'Connell, '52). When tested with a physical source, its output is clear and stable far beyond the requirements of present physiological experimentation. The frequency of its breakers (75 cycles per sec.) does not appear in curves taken with out recorder, a G. E. photoelectric instrument, which has a response time of .04 seconds.

Occasionally, the eyes were stimulated by sudden illumination of the room; but for the most part projection of bright areas upon a screen before the animal was used. In some experiments, real rather than projected objects were presented. The objects were kept stationary in most cases, but they were slowly moved across the field in certain experiments.

RESULTS

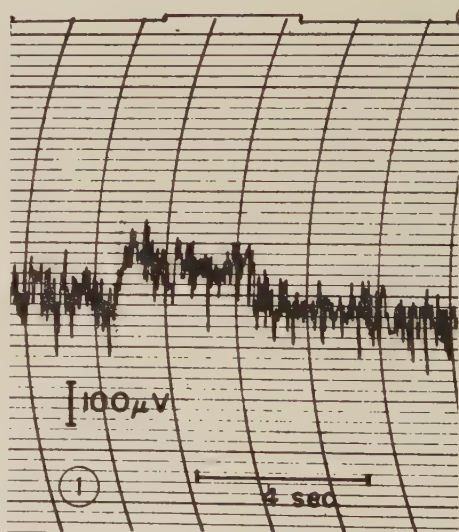
We recorded from the visual projection area of 21 cats. One animal gave no responses, in the case of two only a few convincing records were obtained. From the remaining 18

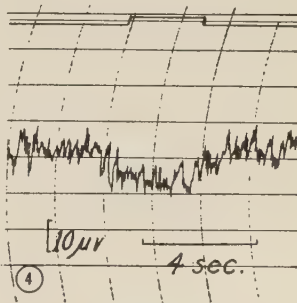
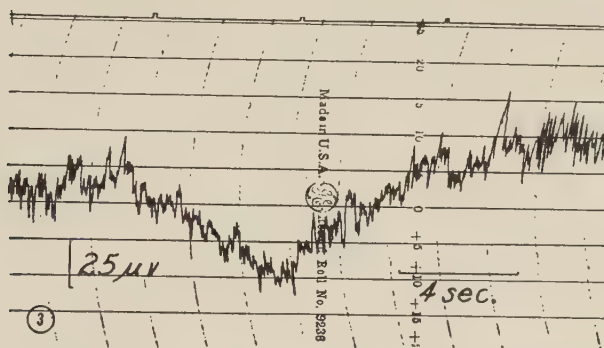
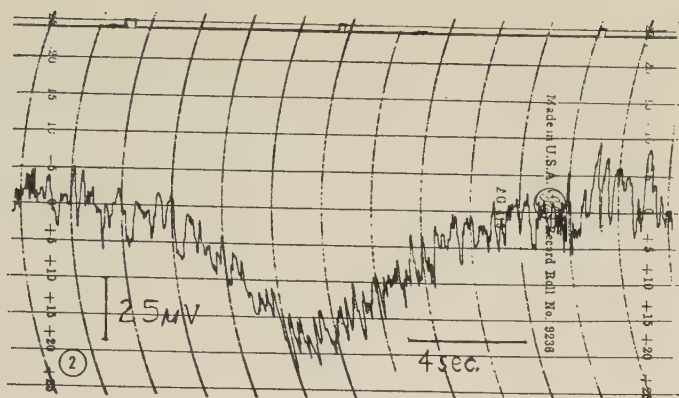
cats clear responses could be registered for considerable periods, although in this group the length of such favorable periods also varied. We do not know the cause of the individual variations. They occur even when, in every respect, preparations seem to be handled in a standard fashion. The records of visual currents to be shown in the present report were taken from 10 preparations.

We found again (*cf.* Köhler, Neff and Wegener, '55) that stimulation establishes no cortical currents under degrees of anesthesia by which some other functions of the brain are little affected. But deep anesthesia is surely not the only factor which tends to reduce or to prevent the responses. When anesthetic action has largely subsided, there may still be periods during which a preparation seems to be useless. Generally speaking, the cat is simply asleep under these conditions, and nothing could be more impressive than the rapid transformation of the base line which occurs when now the well-known arousal reaction is brought about. It seems to us an important observation that, once the base line has changed in this fashion, records of visual currents can usually be taken without any difficulty.

In the following, records of the cat's visual currents will have to be compared with those of auditory currents in the same species, on the one hand, and with records of

Record 1 Auditory current of cat d. Response to a loud tone. Active electrode placed on dura over middle ectosylvian gyrus, grounded electrode on neck. In this and in all following records, a deflection upward indicates surface-negative polarity under the active electrode. Operation performed under nembutal.





Records 2 to 4 Visual currents of human subjects. Record 2: response to vertical grey bar, one inch wide, which slowly moves in horizontal direction before black background. Active electrode placed on intact head over cortical fovea, grounded electrode on vertex. Three marks on top indicate: first appearance of object on one side, coincidence of object with fixation mark, and disappearance of object on other side. Note that current is strongest when object passes the fixation mark, and its cortical representation the active electrode. In the outside circuit the current flows from the active to the grounded electrode. Record 3: visual current of another human subject. Response to vertical grey bar, three quarters inch wide, which moves in horizontal direction before white background. Electrodes placed as in record 2. In spite of reversed brightness relation, shape and direction of response are the same as in 2. Note after-potentials following the primary response. Record 4: visual current of a third human subject. Response to a black vertical bar (stationary) shown slightly to the left of the fixation mark before white ground.

human visual currents, on the other hand. We will therefore now show one curve taken from the middle ectosylvian gyrus of one of our cats (record 1) and, since comparison with human visual records is particularly important, several curves of the latter kind (records 2 to 4). When the auditory record was taken, one of us sang a loud tone. Although the cat's response is not strong, it is perfectly clear. It resembles many curves of our earlier investigation. Its polarity is surface-negative.

The human visual responses were recorded by Köhler and Wegener ('55) in connection with their work on human auditory currents. The active electrode was attached over the cortical fovea, the grounded electrode to the vertex. When record 2 was taken, a vertical gray bar (one inch wide) slowly moved across the visual field before a black background, while the subject fixated a faint mark in the middle of the field. The room was fairly brightly illuminated. The form of the response is typical of records taken under similar conditions (Köhler, '51; Köhler and Held, '49; Köhler, Held and O'Connell, '52). A deflection in the positive direction begins as the object first appears on one side of the field (first mark on top); it grows while the object moves toward the fixation mark and its cortical representation approaches the active electrode (second mark); and it decreases again while the object moves away from the fixation mark on the other side, until it disappears (third mark).

The response shown in record 3 was obtained under similar conditions, excepting that in this test a gray bar ($\frac{3}{4}$ " wide) moved before a white background. The reversal of the brightness relation between the object and its background has no effect upon the polarity of the response as registered. The simple explanation of this fact in terms of physics has been given elsewhere (Köhler, Held and O'Connell, '52). Under conditions of presumably monopolar recording from a distance nothing else is to be expected.

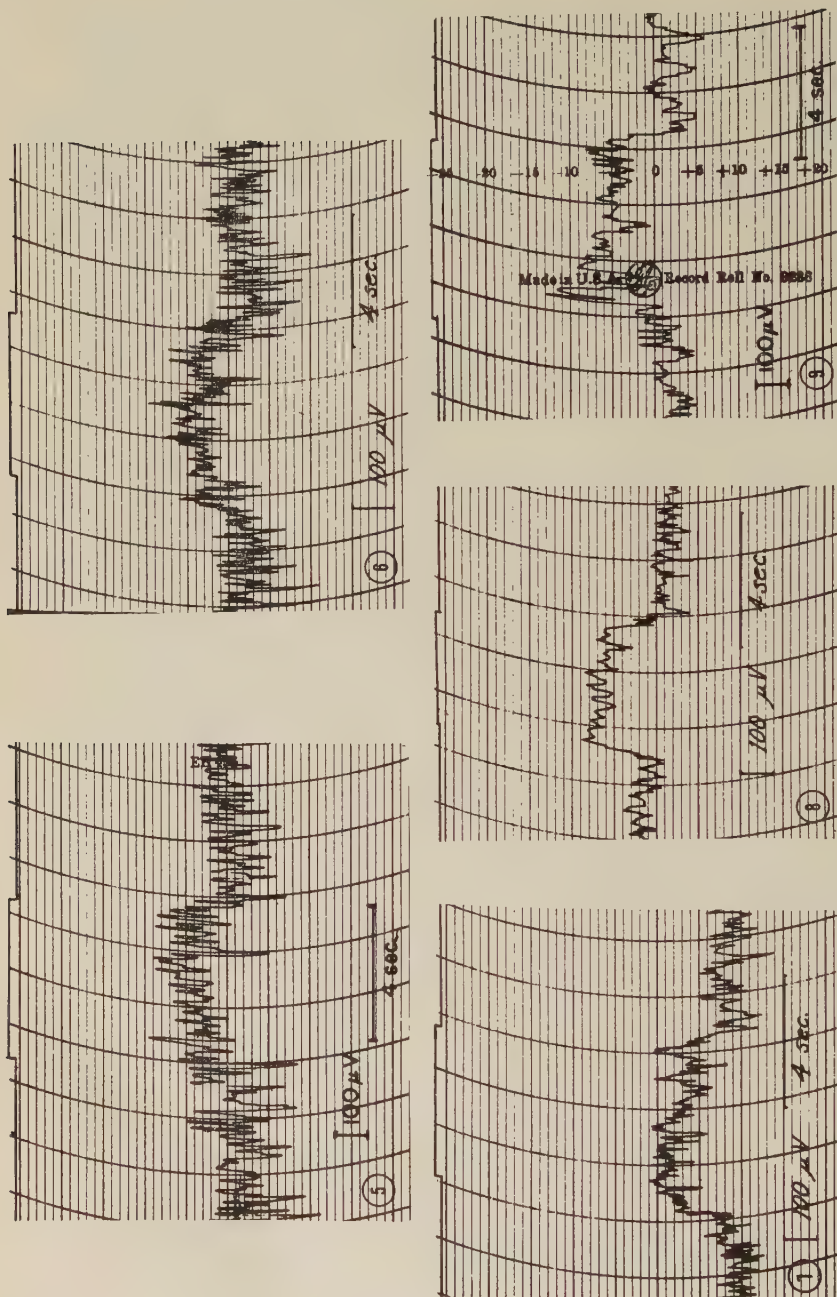
When record 4 was taken, a stationary black bar (one inch wide) was shown before a white background, slightly to the left of the fixation mark. The size of the response is small.

Responses to stationary objects are generally weaker than those to moving objects. As an explanation, it has been suggested that, in the former case, the currents are far more likely to reduce their own intensity by establishing anelectrotonic obstructions in the tissue. It is to be noted that in the present record a black object before a white background again causes a flow of the same polarity as is found with the opposite brightness relation.

We now turn to records of visual currents obtained from the brains of cats. Records 5 to 9 were taken from five different preparations when, in a previously semi-dark room, circumscribed bright areas of rectangular shape were presented for several seconds. These areas appeared at distances varying from one to about three yards. The wick of the active electrode was placed on the dura over the representation of the left or the right half of the *area centralis* and (in one cat) over both. The grounded electrode made contact with the neck.

The size of the present responses varies from about 120 to 180 μ V at greatest intensity. The deflections have the upward direction. It is thus confirmed that, contrary to expectations derived from human visual currents, the cat's visual currents flow through the active tissue from the surface toward the interior. This is the same polarity as that of auditory currents both in the cat and in man. In all our records from the visual projection area of the cat, the deflection caused by steady stimulation has this direction.

Visual currents have been obtained when the visual angle corresponding to the bright surface before the cats was, in both directions, greater than 90° , but also when, both vertically and horizontally, this size amounted to only a few degrees. We often used such brightnesses as 26 m μ L for the bright area and about .3 m μ L for its environment, or also 54 m μ L for the former and 5 m μ L for the latter. With a good preparation, much lower brightnesses and brightness ratios may give clear results. We refrain from mentioning precise measures in each case because such data would be misleading. They would suggest a level of exactness which investigations in this field cannot yet attain.



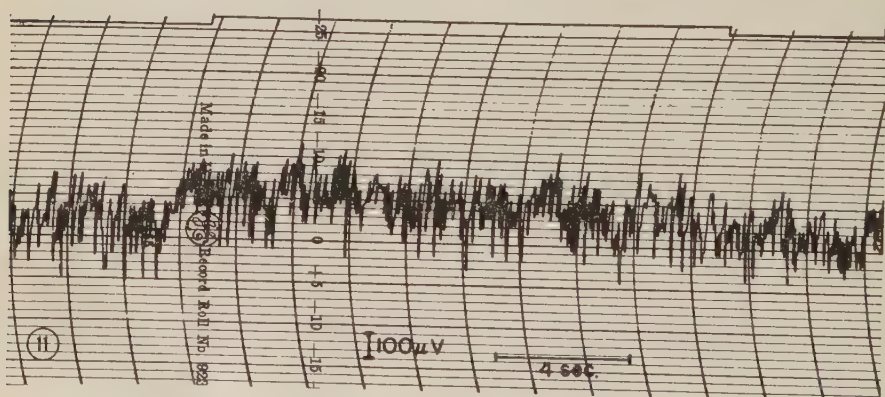
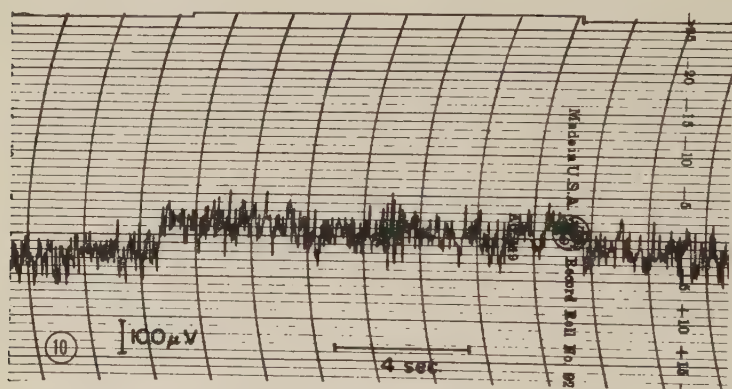
Records 5 to 9 Visual currents of cats v, t, j, f and d. Responses to bright projected rectangles. Here and in following records (excepting records 31 A to C), the active electrode is placed on cortical representation of *area centralis* or on slightly anterior parts of visual cortex, the grounded electrode on neck. In record 5, operation performed under pentothal, in all others under nembutal.

In comparison with the condition of the preparations at the time of a test, such objective parameters as area and intensity of the stimulus have only limited significance, and we are only beginning to discover how that condition can be kept favorable and more or less constant. To a degree, this difficulty can be overcome by changing the stimulating conditions while a record is being taken. For instance, we sometimes abruptly increased or decreased the intensity of the light during a given test, and found that corresponding changes of the current tend to be small, a fact which is in line with Fechner's law.

The form of the present responses greatly resembles that of the cat's auditory currents. The similarity goes so far that, even after much experience with the cat's auditory and visual currents, we would often not be able to decide whether a given curve is of one or the other origin. Variations of the form from one record to another are also about the same in both cases. Occasionally, visual currents reach their greatest intensity very fast (cf. record 8); in other curves, their full size develops more slowly (cf. record 7). In record 9, a quite abrupt first deflection occurs only after an appreciable latency time. There are also considerable variations in later parts of the responses. Generally speaking, the currents begin to decrease before the end of stimulation. On the other hand, however, they often continue to flow for a short time beyond this moment. In fact, this holds for records 5 to 9 without exception.

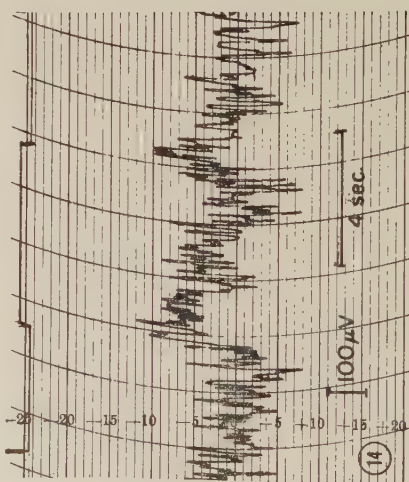
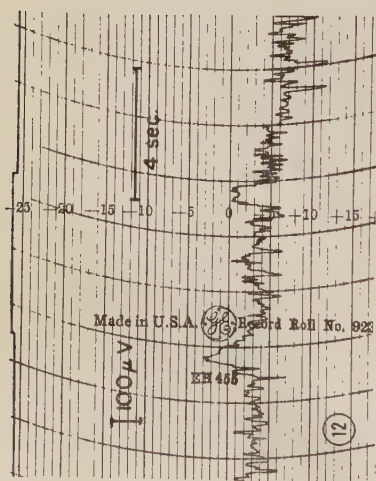
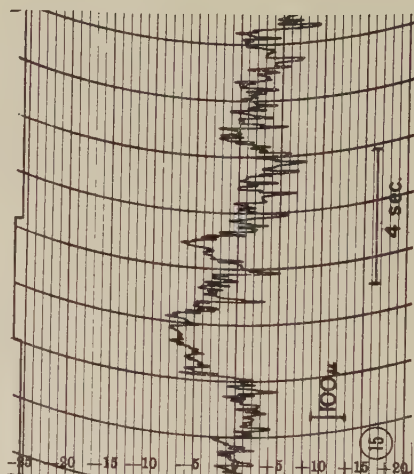
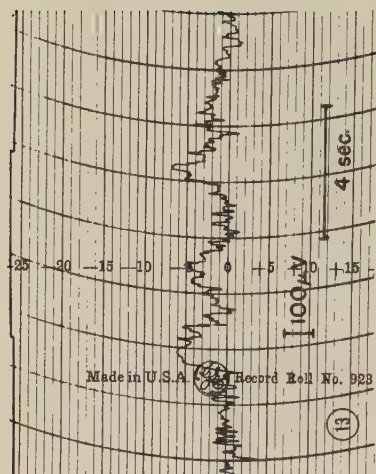
The temporal characteristics of the currents are further illustrated by the following records. Stimulation was sometimes continued for long periods. Under these conditions, the responses of good preparations persisted until the end of such periods. When record 10 was taken, a ceiling lamp illuminated the previously fairly dark room during the time indicated on top of the record. From the location of the cat, the lamp itself was not visible, and there was no strongly illuminated surface before the animal. Nevertheless, the record shows a clear, though weak, response which persists for the whole period of stimulation, that is, for about 11.5 seconds. On closer inspection, this response will be found to decrease slowly after the

first few seconds. A short secondary deflection upward seems to follow the end of stimulation (cf. records 12 to 15, below). Record 11 was obtained when a similar test was done with another cat. In this case, however, a bright rectangle projected upon a screen before the animal appeared for approximately 16 seconds. The response, which is stronger than that of the preceding record, slowly decreases from its size during the first seconds, but it also persists for the whole period of stimulation. (Both records ought to be inspected by looking along the curves in the direction of time. With this procedure, the temporal course of responses of long duration is far more clearly discernible.)



Records 10 and 11 Quasi-steady currents maintained as responses to light shown for 11.5 sec. (cat d, operation under nembutal) and for 16 sec. (cat u, pentothal).

However, in the cat, visual currents often decrease very fast, just as do the auditory responses. Many curves show such a rapid descent. A particularly striking example is record 12. Here, a fairly steep first ascent to a level of about 150 μ V is very soon followed by a fast return to zero level so that in later parts of the stimulation period the response seems to have disappeared. As has been explained elsewhere (Köhler and Wegener, '55, p. 33), this form of the registered response need not mean that, when no deflection is left, the cortical current itself has ceased to flow. Cortical currents are likely to change their distribution continually as a consequence of the electrotonic obstructions which they establish. The intensity of the registered flow must vary correspondingly, because a change in the distribution of the current will generally be accompanied by a change of the potential at the active electrode. When the electrode is not optimally placed, electrotonic effects may raise the potential at the active electrode, and thus intensify the registered current (electrotonic spread). But just the opposite may also happen, and must always happen, when electrotonic action weakens the flow which reaches the electrode. In fact, the potential at the active electrode may thus be lowered to zero, while nevertheless a current of some size continues to flow through the source. This is perhaps the right interpretation not only of curves such as the present record but also of other fluctuations which the intensity of registered responses tends to show under conditions of constant stimulation. For instance, in record 13 (which was taken from the same cat as record 12, and only a few minutes later) a first rapid descent of the response is almost immediately followed by a second deflection upward, which then also disappears. It is not necessary to assume that this form of the registered current demonstrates a rhythmic behavior of the current as such. Variations of the electrotonic pattern established by this current may cause semi-rhythmic changes of the potential at a given point while no fluctuations of comparable size occur in the intensity of the flow as a whole. The over-all intensity of the current must steadily decrease as electrotonic



Records 12 to 15 Less persistent responses recorded from cat f (nembutal), 12 and 13; cat z (pentothal), 14; and cat j (pentothal), 15. Note the after-potentials in all four records.

obstructions grow and expand. At any rate, the present curve and fairly frequent similar records do not prove that the intensity of the total flow slowly oscillates.

In addition to a rapid decrease of the registered response, record 12 exhibits another phenomenon; almost immediately after the end of stimulation a new deflection upward occurs. This is by no means an accidental deviation of the base line; it is found in a great many records, and undoubtedly represents an after-potential. Records 13 to 15 may serve as further examples. After-potentials are, of course, most easily recognized when the original response has disappeared during the stimulation period so that the new deflection is clearly separated from the first. This is the case in records 12 to 14. The distinction becomes more difficult when the original response remains intense to the very end of stimulation. Occasionally, one obtains curves in which the current seems to persist considerably beyond the stimulation period. Actually, in some such curves the original current may not continue to flow far beyond the end of stimulation, but it may then fuse with the beginning of an after-potential which follows immediately. In record 15 this has almost happened. If, in this curve, the original response had been only slightly stronger at the end of stimulation, an interpretation of the registered curve as consisting of the original response and an after-potential would have seemed arbitrary.

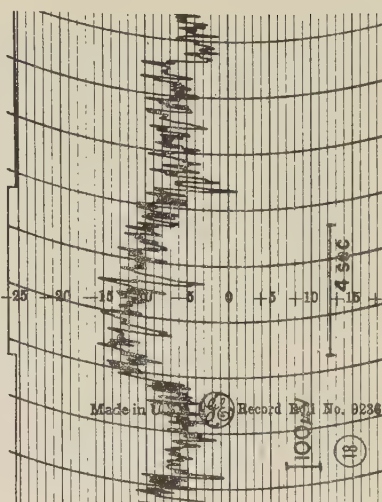
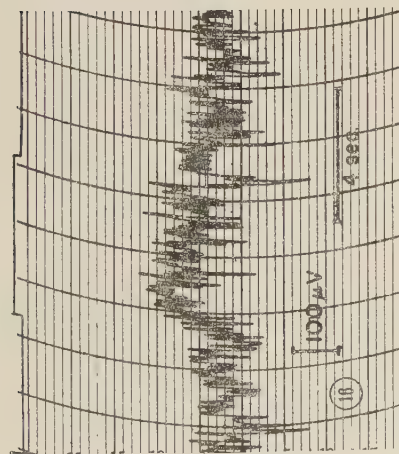
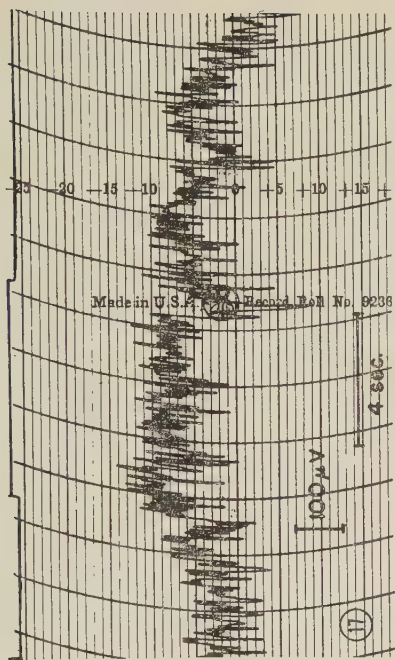
Available records of human visual currents show many examples of after-potentials (cf. record 3, page 5). As a matter of fact, in such curves a first after-potential is sometimes followed by a second. In man, too, such deflections after the stimulation period occur in the same direction as the original responses. Thus they indicate a surface-positive polarity at the source, while the after-potentials of the cat's visual currents have the surface-negative direction. Incidentally, both in man and in the cat, visual responses seem to exhibit after-potentials more regularly than auditory responses do.

Another phenomenon occurs far more frequently and strikingly in records of auditory currents. The short but strong

positive deflection with which many auditory responses begin (and sometimes also end) is not often seen in visual records. Even when such "evoked potentials" are merely caused by telephone clicks, they must be regarded as highly characteristic auditory reactions. Similar waves may also appear in visual records taken from cats or human subjects; but, on the whole, they are smaller and of a less abrupt character in visual than in auditory responses. On the other hand, some visual responses recorded from cats are *followed* by short and strong positive waves. Records 16 and 17 were taken from the same cat, the second only one minute after the first. In record 16, the visual response as such is comparable to many others; but, when stimulation is interrupted, a sharp deflection in the positive direction follows at once. There seems to be no difference between this wave and those which precede and follow many auditory responses. In record 17, a short deflection downward upon the onset of stimulation may perhaps be regarded as an evoked potential; but a far more impressive positive swing follows the end of stimulation. In this curve, the short positive wave is clearly followed by two after-potentials in succession.

Even the striking positive deflections shown in these curves may have to be regarded with some caution for the following reason. Fairly abrupt waves of this kind appear in our curves not only at the end of stimulation but also, and by no means infrequently, at various times during stimulation by an unchanging bright area. In record 18, for instance, such a deflection occurs in the very middle of the stimulation period. If this deflection coincided with the onset or the end of stimulation, it would invite comparison with similar deflections registered at the beginning and at the termination of auditory stimulation. In the present case, however, such a comparison would seem to be unwarranted. Hence, we cannot be entirely sure that it is justified in the two preceding instances.

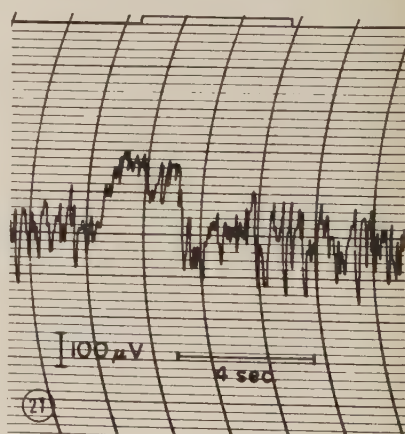
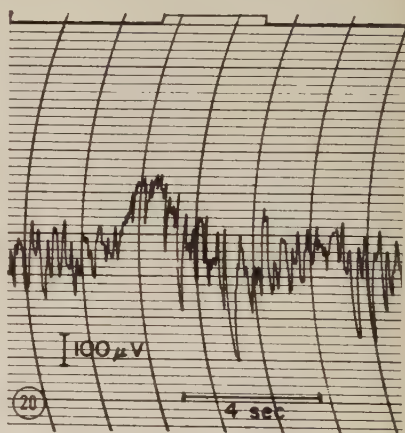
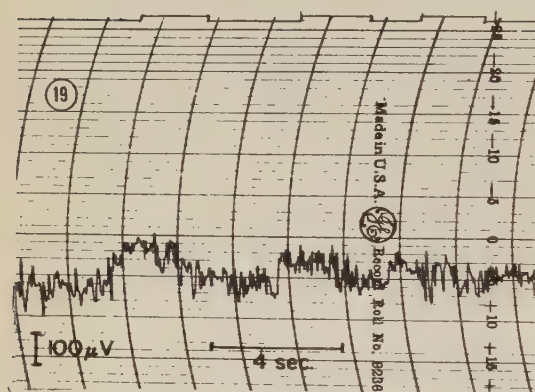
Records 2 to 4 have shown that the polarity of human visual currents is not affected by a reversal of the brightness relation between the object and its background. Whether the object or



Records 16 and 17 Responses followed by sharp positive deflections (cat w, pentothal). Note after-potential in 17.

Record 18 shows that sharp positive deflections may also occur during stimulation (cat j, pentothal).

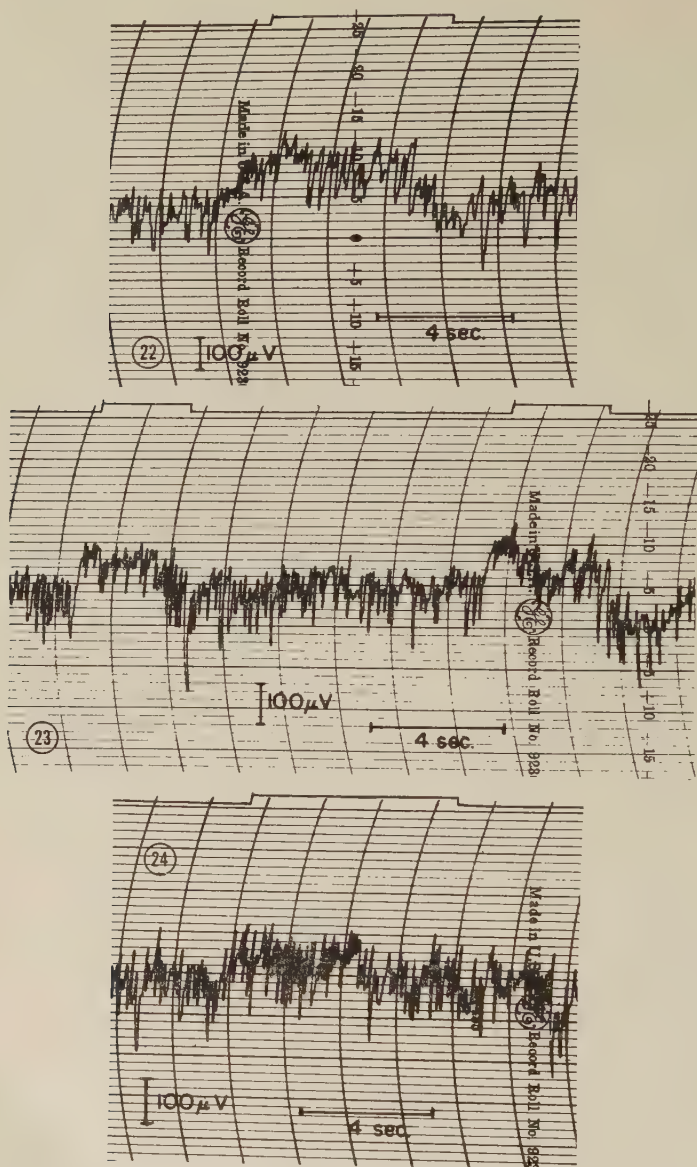
its environment is brighter, the registered currents always indicate a surface-positive potential at the source. But the argument which shows that nothing else can be expected from the point of view of physics is quite general (Köhler, Held and O'Connell, '52, p. 313 f.). It applies to any visual currents irrespective of their polarity. Currents from the cat's visual cortex must therefore exhibit deflections in the upward direction both when the object is brighter and when it is darker than its background. Records 19 to 21, prove that this is actually true. When record 19 was taken, fairly intense light had for some time been projected upon a large screen before the



Records 19 to 21 Responses to the appearance of sharply outlined shadows within a large bright field. In 19 (cat d, nembutal), the shadow is presented three times in succession. In 20 (cat j, pentothal), the response decays rapidly, and is followed by a sharp positive deflection; in 21 (cat j, nembutal), it ends abruptly in the middle of the exposure. All responses have the surface-negative polarity.

cat. In this bright field a sharply outlined area was then suddenly darkened three times in succession. It will be seen that the curve exhibits three weak but clearly recognizable reactions to the presentations of the dark area, although the third current is very faint indeed. The direction of these responses corresponds to a surface-negative cortical potential. Records 20 to 21 were taken from another cat but under the same conditions as record 19. There can be no question as to the polarity of the fairly strong deflections which these curves exhibit. In record 20, the registered current decreases rapidly; in 21 it ends abruptly long before the dark object disappears. Incidentally, in record 20, the disappearance of the object is once more followed by a strong positive wave (cf. records 16 and 17, above); but again, a similar positive swing also occurs in the middle of this response.

Many human visual records have been taken when real objects in highly illuminated rooms rather than projections in dark rooms served as stimuli. In fact, Köhler, Held and O'Connell ('52) found that, in man, clear visual responses can be more easily obtained in the former condition, and practically all visual currents which have since been registered from human heads have been recorded in this fashion. When taking records of visual currents from the cat's brain, we have mostly used projected stimuli. In order to avoid the impression that this is a necessary procedure we will now show a few records in which real objects served as stimuli also in the present study (records 22 to 24). Brightness differences between the objects and their background were fairly small under these circumstances. When record 22 was taken, a strong lamp, not visible to the cat, illuminated the field, a large part of which was a black surface. Before this surface a piece of white cardboard was suddenly shown, and equally suddenly removed after about 5.5 seconds. The response, which has, of course, the negative direction, is perfectly clear. It persists until the end of the stimulation period. Sometimes we presented gray rather than white objects before a black background and thus re-



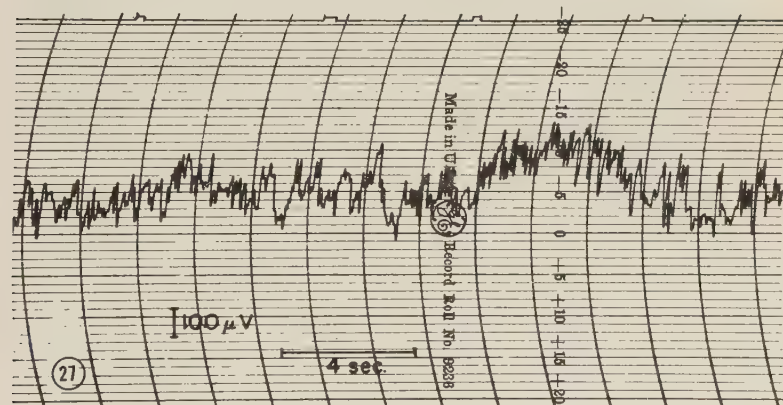
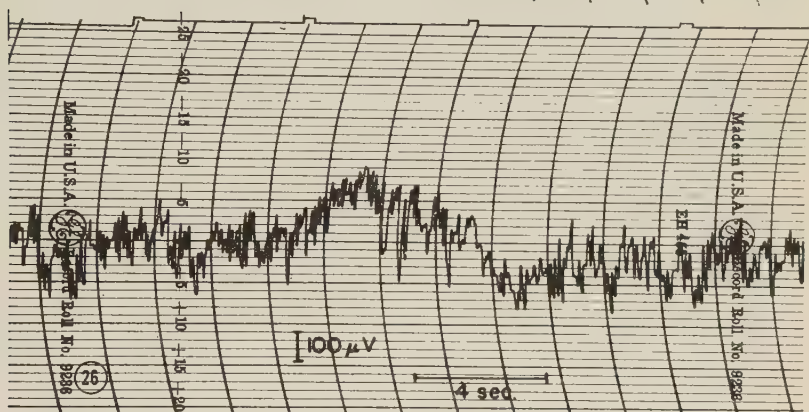
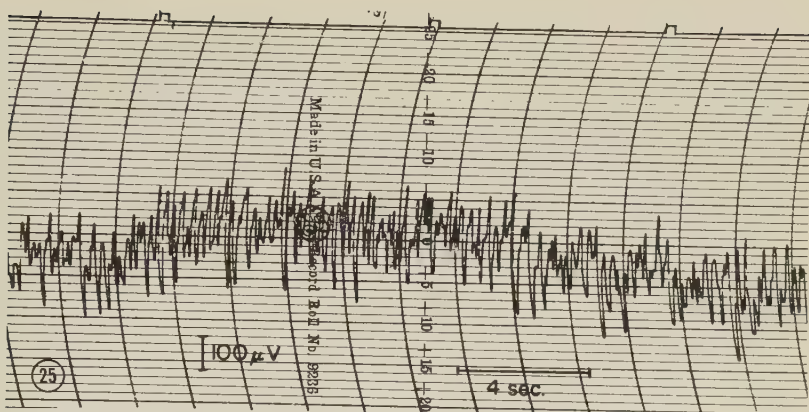
Records 22 to 24 Responses to real objects shown in bright illumination. In 22 (cat j, pentothal), a piece of white cardboard is shown before a black background; in 23 (cat w, pentothal), a grey object appears twice before a black surface; in 24 (cat w, pentothal) a black object is shown before a white background. All three responses have the surface-negative polarity.

duced the brightness difference still further. Such an object, a gray circle, the diameter of which corresponded to a visual angle of 30° , was shown twice in succession when record 23 was obtained. The two responses are well defined. The second probably makes the impression of continuing beyond the end of the exposure period only because the end of the primary response is not clearly separated from the beginning of an after-potential (cf. record 15, above). In experiments with real objects, the dark background can, of course, again be replaced by a white field, and the bright object by a black disc. Record 24 shows that, when this is done, there is once more a response of the same polarity as if the object were bright and the background dark. In the present instance, the registered current is weak, and it does not persist to the end of the exposure time. On the whole, it is our impression that, while operating with real objects is the best procedure in the study of human visual currents, it offers no similar advantage in work on the cat's visual responses.

It will be remembered that human visual responses have a particularly characteristic shape when the object slowly moves across the field. Under these conditions, the current tends to grow steadily as the object approaches the fixation mark, and to decrease again as the object moves toward the periphery on the other side. For the most part, the deflection reaches its greatest size when the object passes the fixation mark; the maximum is often quite marked (cf. records 2 and 3, above). This shape of the responses appears readily understandable in terms of the location of the various parts of the human visual cortex in their relation to the place of the active electrode, because in man only the cortical fovea lies immediately under the skull, and is therefore nearer the active electrode than are more peripheral parts of the striate area. In similar tests with cats, we showed bright projected bands in a horizontal orientation, which moved in the vertical direction. Geometrical relations between the various parts of the cat's visual projection area and an active electrode in contact with its

center can hardly be compared with those which obtained when records such as 2 and 3 were taken from the heads of human subjects. We therefore did not expect that responses would have precisely the same form as they have in man. In actual fact, the sharp maximum which is often found in the latter case seldom occurred in records taken from the visual cortex of cats. The curves shown in records 25 to 27 may serve as examples. In records 25 and 26, the wick of the active electrode was placed on the dura over the representation of the *area centralis*, and when the bright moving band passed the middle of its course its height corresponded approximately to the direction of the cat's eye. The movement had the downward direction. In record 25, the three marks on top indicate the first appearance of the band, its arrival at a position near the middle of the movement, and its disappearance. It will be seen that the response begins almost immediately after the appearance of the band, and that it then persists for the larger part of the exposure without essential changes of its size. A more frequent form of the response is shown in record 26. The four marks on the top of this record indicate, apart from the first appearance and the disappearance of the band, two positions between which it passed the middle of its course and at the same time the direction of the cat's eye. In this curve the length of the response is greatly reduced. The moving band now affects the active electrode only while the distance of its cortical representation from this electrode is short. Moreover, within this range the response clearly exhibits a gradual growth to greater size, and then an equally gradual descent. To this extent it does resemble responses of the human visual

Records 25 to 27 Responses to slowly moving objects. In 25 (cat v, pentothal), a bright horizontal band of considerable width moves vertically downward across the field. Note the accentuation of the cortical rhythm upon the appearance of the bright object. In 26 (cat m, pentothal), a much narrower band causes a response only within a limited part of the exposure period; the location of the response corresponds to the location of the active electrode. In 27 (same cat), the movement field is raised until its lowest third occupies the place which its middle occupied before; the response is correspondingly displaced within the record.



Records 25 to 27

cortex which are taken under more or less comparable conditions.

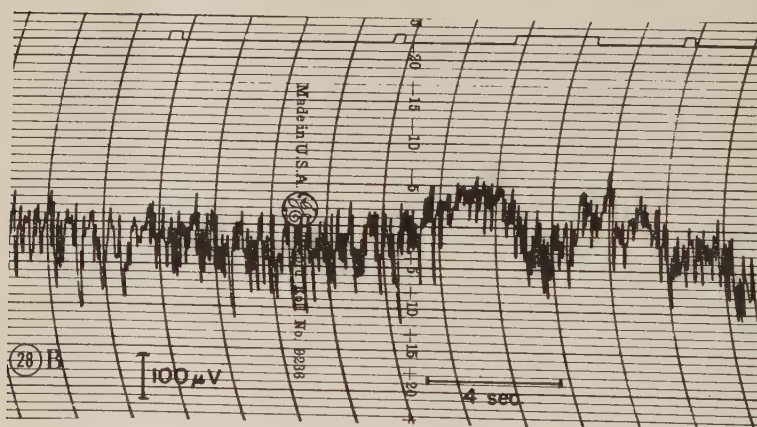
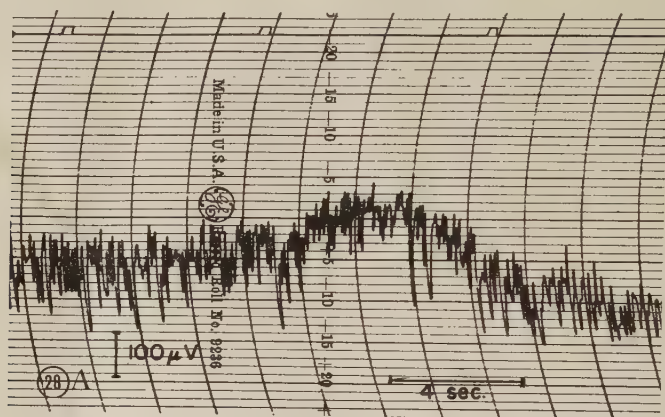
The shape of the present curve suggests that the specific location of such circumscribed responses to moving objects is related to the position of the active electrode and thus to facts of localization within the cat's visual projection area. In order to test this assumption one can, for instance, vary the location of the movement field in relation to the eye, or also the spatial characteristics of the moving object. We have repeatedly done experiments of this kind. For example, if a particular position of the response within the exposure period is a matter of specific localization within the visual cortex, this position must shift in a predictable fashion when now the movement field is lowered or raised as a whole. Thus in record 26 the response is located in the middle of the exposure period. This response should shift toward the end of the period when, in another test with the movement downward, the movement field is raised as a whole until its lowest part occupies the place previously occupied by its middle part. In record 27, which was taken from the same animal but under these changed conditions, the response is obviously displaced toward the end of the exposure period; that is, in relation to the eye and to the place of the active electrode, it occurs in the same place as before. Similarly, if in a given test the response has occurred at the end of the exposure period, it must occur in the beginning of this period when, under otherwise identical conditions, the direction of the movement is reversed in the next test. When this experiment was performed, the result again agreed with the prediction. Obviously, the location of responses to moving objects can be interpreted in terms of cortical localization. So far as the moving object is concerned, one expects the response to be more or less circumscribed depending upon the width of the moving band. Actually, the response in record 25 already demonstrates that this is true. For when it was taken, the visual angle corresponding to the width of the moving band was far greater than it was in the case of records 26 and 27. This is probably the reason why, in the former case, the re-

sponse extends over most of the exposure time while it is concentrated about the center of the movement in record 26.

We are not convinced that similar tests will invariably be successful. In experiments of this kind, decisions must be derived from a comparison of several records. This procedure presupposes that the preparation remains in a more or less constant condition from one test to the next. This is surely not always the case. Moreover, in experiments which deal with matters of localization within the visual projection area, the eyes must be assumed to have a constant direction. Now it is true that, so long as appreciable effects of the anesthetic are left, cats do not often move their eyes. But they may do so occasionally; and, when this happens, records can, of course, no longer be interpreted in terms of cortical localization. It remains true, however that in almost all tests of this kind which we performed results agreed with predictions, and, more particularly, with expectations derived from the work of Talbot and Marshall ('41).

Responses to moving objects can be used for a simple but probably important demonstration. Repeatedly, we have referred to anelectrotonic obstructions by which cortical currents weaken themselves. If this concept has been properly used, the response to an object which first moves across the field ought to be rapidly reduced when the movement is suddenly interrupted; for the polarization of the tissue by the current of the object must be greatly enhanced when the cortical representation of the object occupies a given place for some time. Elsewhere it has been shown that, in the case of human visual currents, this effect actually occurs. We have had no difficulty in obtaining the same phenomenon when registering the visual currents of cats. When record 28A was taken, a bright horizontal band moved downward without interruption. The response, which is restricted to the second half of the exposure, is continuous within this region. Record 28B, which was taken from the same preparation, shows the response to the same conditions, excepting that now the movement was suddenly

interrupted at a point where, in the preceding test, the deflection had just attained its greatest size. After 2.5 seconds, the band began to move again. (The continuous deflection of the signal line on top corresponds to the time during which the movement was interrupted.) It will be seen that within about a second after the object has become stationary the current



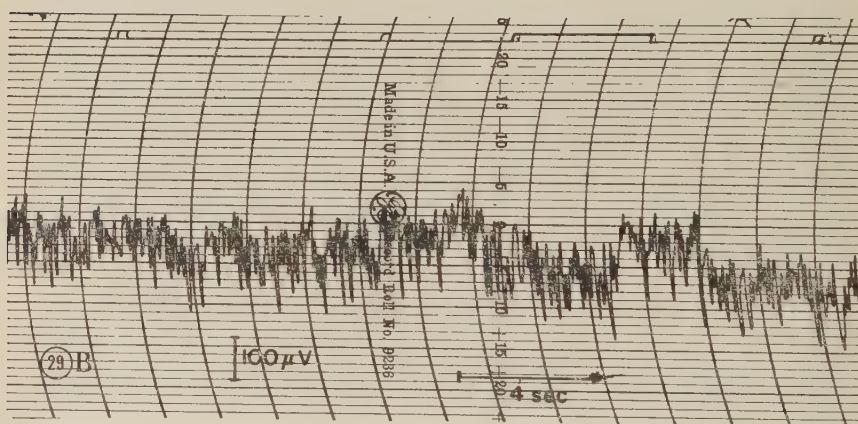
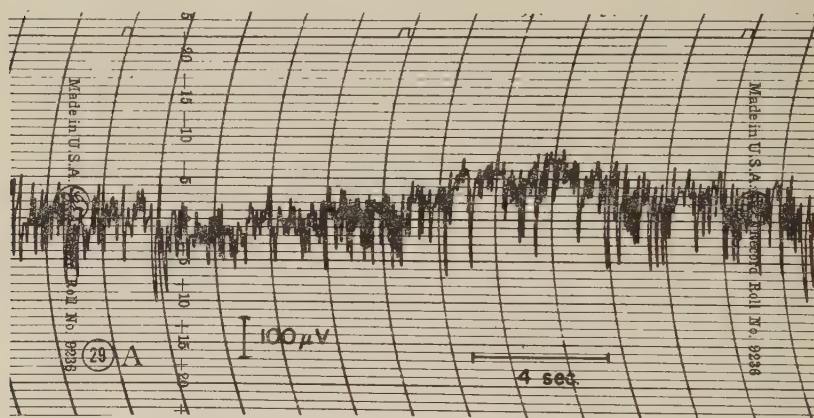
Records 28 A and B Reduction of response to a moving object when movement is temporarily interrupted. In 28 A (cat t, pentothal), the object moves without interruption; the response occurs in second half of exposure. In 28 B (same cat), the movement is interrupted during period indicated on top; the response is greatly reduced during this period.

begins to decrease, but that it soon recovers when the band resumes its movement. This record should be compared with record 28 of our earlier paper (Köhler, Held and O'Connell, '52, p. 316), which was taken under comparable conditions from the head of a human subject. Apart from the fact that in the latter record the registered current indicates a surface-positive polarity, the reduction of the response during the stationary phase of the exposure is equally obvious in both cases. Incidentally, in the beginning of record 28B, the character of the base line clearly changes upon the appearance of the bright object; the frequency of the spontaneous rhythm is suddenly increased at this point.

The effect shown in record 28B can also be demonstrated when the bright band on a dark background is replaced by a dark band which moves across a bright field. This was done in our next experiment (records 29A and B). The response in record 29A is weak but clearly discernible as a slow continuous deflection upward during the second half of the movement. (Once more, the reversal of the brightness relation between the object and its environment has not affected the polarity of the registered current.) When record 29B was taken from the same preparation, the movement of the dark band was interrupted for 4 seconds in a position where, in the preceding curve, part of the response was located. In the present record, this response is still recognizable before and after the stationary part of the exposure; but during this part it has disappeared. We hesitate to believe that the reduction of the cortical current when the object does not move can be explained as a result of "retinal fatigue." The great speed of the reduction can hardly be understood in such terms. On the other hand, this speed seems to us entirely compatible with the view that the reduction is caused by the increase of local polarization (anelectrotonus) which must follow the interruption of the movement.

Are the responses shown in our records actually currents of the visual cortex? We have sometimes been asked whether

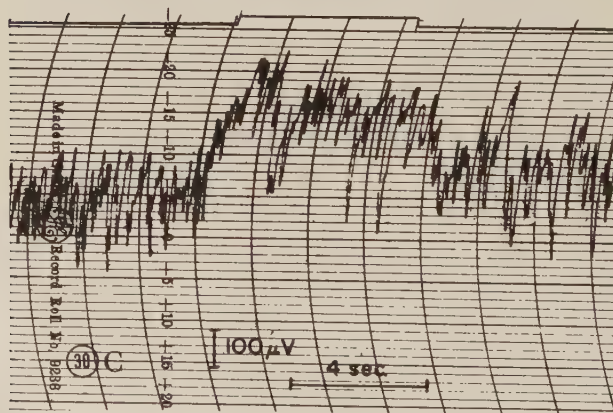
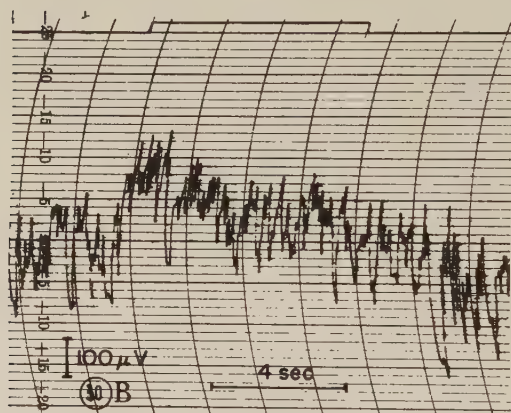
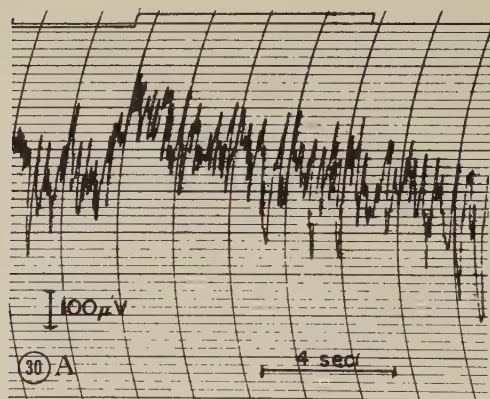
such curves could not be explained in terms of peripheral factors. *Prima facie* there seem to be two possibilities of this kind: it might be suggested either that the registered responses are related to eye movements which follow the onset of stimulation, or that they are electric reactions of the retina to stimulation by light.



Records 29 A and B Experiment 28 repeated with a black band moving across a bright field (same cat). In 29 A, the movement is not interrupted; the response occurs in second half of exposure. In 29 B (same cat), the movement is interrupted during period indicated on top; the response is sharply reduced during this period.

As to the first suggestion, we have already mentioned that the records shown so far have been taken under conditions which do not exclude eye movements. Such movements can influence the potential at the active electrode by changing the location of retinal images and thus of their cortical counterparts, but also by establishing currents of their own. If there have been such influences, they must have been rare. This follows from the results of the experiments which have just been described. Circumscribed responses to moving objects are, and remain for long periods, localized in a fashion which could hardly be understood if the cats often moved their eyes. It is quite true that, in the experiments done by Köhler, Neff and Wegener, eye movements of the cats have actually been recorded; but the cats used in the present investigation were invariably kept under somewhat deeper anesthesia than the preparations used in the earlier study. Even so, we decided to subject the issue to experimental tests. Doctor S. Kuffler of the Wilmer Institute was kind enough to immobilize the eyes of one of our cats by attaching them firmly to their anatomical environment. The procedure is well known, and therefore need not be described here.

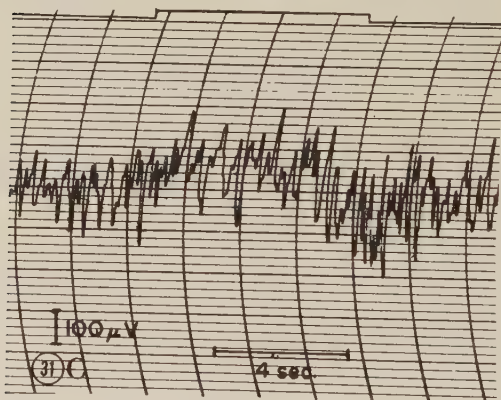
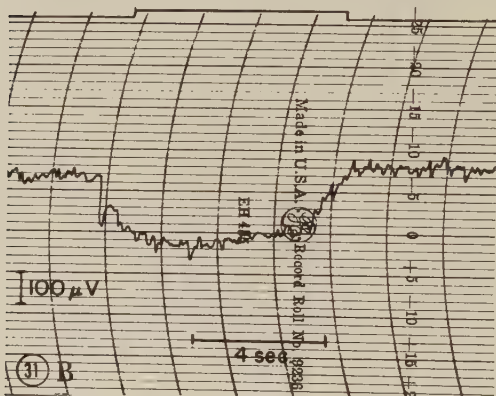
When the operation had been completed, not the slightest visible movements of the eyeballs could be produced by pressure in one direction or another. In our tests, only the right eye was used; the left eye remained closed. The active electrode made contact with the dura over the representation of the *area centralis* on the right side; the grounded electrode was attached to the neck. Bright projected objects, both stationary and moving, served as stimuli. They were presented in the direction given to the eye by the operation. When the effects of anesthesia had sufficiently subsided, we took 33 records altogether; in 30 cases we obtained clear responses of the same kind as are recorded from cats whose eyes are not immobilized. (Only the spontaneous rhythm was stronger than it usually is, most probably because, soon after the operation, application of picrotoxin had appeared advisable.) In view of this finding,



Records 30 A to C Responses to stationary objects when both eyes are immobilized (cat r, pentothal).

it can no longer be maintained that the currents shown in our records are essentially related to eye movements. It will suffice if we reproduce three curves (records 30A-C). In records 30A and B, the size of the responses decreases fairly fast; in record 30C, the primary response seems to be followed by after-potentials. Our results are not at variance with the findings of Riggs, Ratliff, Cornsweet and Cornsweet ('53) who found that, when the image of a thin line occupies a strictly constant retinal position, this line begins to disappear within a few seconds. For the authors also report that this effect is greatly delayed when larger objects are presented. The size of the bright rectangles shown in the present experiments was considerable.

The question whether the responses obtained from the visual projection area are electroretinograms rather than cortical currents can also be answered by simple tests. Quite apart from such tests, it is obviously improbable that the responses are of this origin; because the currents which the retina develops under the influence of light have the cornea-positive polarity. With the electrodes placed over the cortical fovea and on the neck as they were in our experiments, retinal currents would *raise* the potential at the active electrode; in other words, the polarity of such currents would be opposite to the one actually found in our records. Nevertheless, we decided to clarify the situation experimentally, and therefore registered electroretinograms from five of our cats with the grounded electrode attached to the neck, and the active electrode to anterior parts of the head. Results were so uniform that we will show only three records, all taken from the same cat, and all under the same conditions of stimulation. Both eyes were kept open; they were protected by oil, and atropin was used. Adaptation corresponded to a faint illumination of the room. During a first test (record 31A), the wick of the active electrode was placed on the forehead, just above the eyes and in the median plane; the grounded electrode was attached to the neck. The response, immediately recognizable as an electroretino-



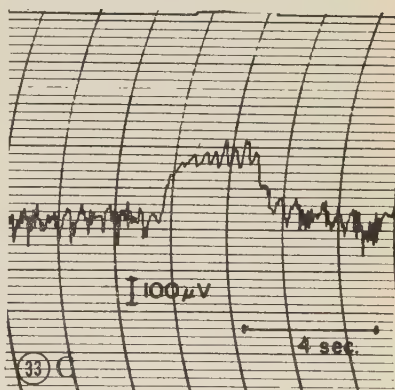
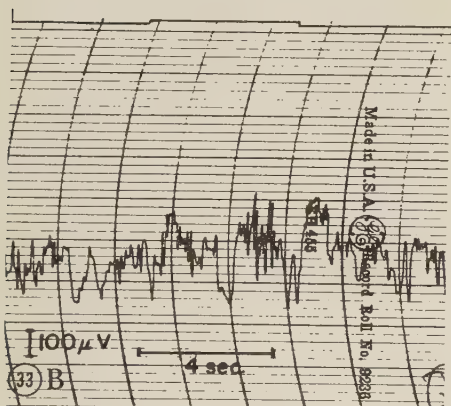
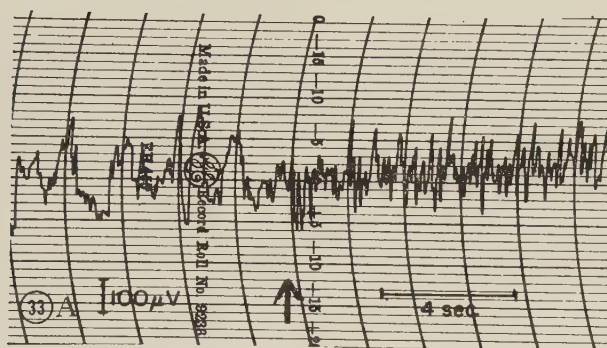
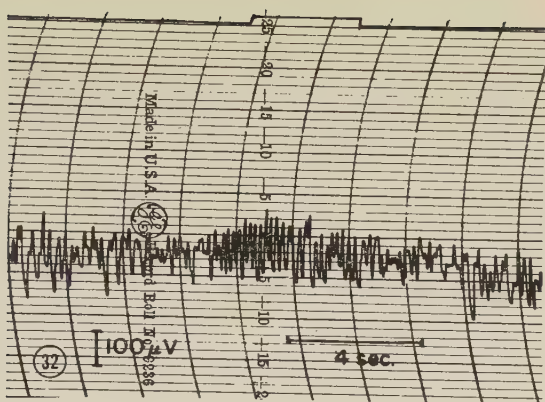
Records 31 A to C Electroretinograms compared with cortical visual current (cat v, pentothal). In A, the active electrode is placed on forehead in medium plane; in B, 1.5 cm farther backward; in C, 2.5 cm behind its first position. In C, the positive polarity of the retinal current is replaced by the surface-negative polarity of the cortical current. Note the cortical rhythm in C.

gram, indicates an intense and persistent positive potential at the active electrode, the c-wave, which is preceded by the well known b-wave. The size of this short wave has probably been reduced by the slow reaction of our recorder. For the same reason, other details such as the a-wave have not been recorded. The next record (31B) was taken eight minutes later, with the active electrode attached to bone 1.5 cm behind its place in the first test. The response, although considerably weaker, is still recognizable as an electroretinogram; it still exhibits the characteristic b-wave. Closer inspection shows that the rapid fluctuations already discernible in record 31A are now more pronounced. In the third test (record 31C), taken three minutes later, the active electrode was placed on bone 1 cm farther back, that is, altogether, 2.5 cm behind its first place. This curve has an entirely different appearance. The spontaneous cortical rhythm is now fully developed, and during the exposure there is a negative deflection, obviously a response of the same kind as was obtained when the active electrode made contact with the dura over the cortical representation of the *area centralis*. Further tests with the same and with other cats demonstrated beyond any doubt that, under the conditions of our tests, electroretinograms can be recorded only within a region distinctly anterior to the area in which the active electrode was placed in our ordinary experiments. Hence, the responses recorded in these experiments cannot be interpreted as electroretinograms.

Two further observations made during our study of the cat's visual currents remain to be mentioned. Both refer to the spontaneous rhythm of the visual cortex, but indirectly also to the nature of the cat's visual currents. In our tests, stimulation of the eyes by light never blocked the cortical rhythm in the way in which light blocks the human alpha waves. We may not have worked under the very best conditions for the demonstration of this effect. We also realize that the cortical rhythm of the cat is reduced by other factors such as opening of the eyes. But, to our knowledge, it has not been proved that under

these conditions the blocking is caused by visual stimulation. Opening of the eyes and keeping them open must be accompanied by sensorimotor processes which might affect the rhythm. Moreover, in our experience, stimulation by light has not only failed to cause blocking; it has, on the contrary, sometimes enhanced the rhythm. A change of this kind was obvious in record 28B; but it also occurred in record 25 and again in record 27, where the rhythm was clearly accelerated during the circumscribed response to a moving object. Or take record 32 which shows a barely recognizable steady response to weak light, but an obvious acceleration and regularization of the rhythm, which begins with the onset of stimulation. Unfortunately, our instruments did not allow us to subject this issue to fully adequate tests, because they do not register waves of higher frequencies than about 20 cycles per second. Thus, if the cortical waves were sometimes accelerated beyond this limit, we could not discover the effect even if the waves were intensified at the same time. It is, of course, known that in the cat's auditory area the cortical rhythm is greatly accentuated by sounds. This has been shown by Bremer ('37 and '43) and by Rheinberger and Jasper ('37).

We had no difficulty in recording another alteration of the cortical rhythm, namely, the arousal reaction. In our cats, the waves of the visual cortex often became large, slow and irregular. When this happened, we found it practically impossible to register visual currents. Not only did the large fluctuations obscure the steady potentials produced by stimulation, but for the most part there was no indication that under such circumstances the potentials were at all established. The slow and large waves greatly resembled those which other investigators had observed when their experimental animals were obviously asleep. It therefore seemed probable that during such periods no visual currents could be recorded for the same reason. If this was true, the well-known arousal reaction had to occur in our cats as soon as certain non-visual stimuli were applied, and it was our hope that, once the cortical waves



Record 32 Accentuation of cortical rhythm under the influence of light (cat t, pentothal).

Records 33 A to C Record A shows arousal reaction (at time of arrow) produced by pinching of ear (cat j, pentothal). Record B shows cortical rhythm during sleep; exposure of a bright object (see signal on top) causes no response (cat f, nembutal). Record C is taken one minute later from same cat after repeated pinching of ear: the same stimulation now produces a clear response.

had assumed the form related to the waking state, the changed condition of the brain would also permit the recording of visual currents. Tests immediately verified these expectations. When the slow waves had developed, we simply squeezed the tail, a paw or an ear of the cat. Almost invariably, this procedure produced the desired effect; it transformed the slow, irregular fluctuations of the base line into the "normal" rhythm (cf. record 33A, where the arrow roughly indicates the moment of non-visual stimulation). When this had happened, visual currents could generally be recorded without difficulty. Between the recordings 33B and C, no more than a minute elapsed. During this period, one ear of the cat was repeatedly pinched. Record 33B shows the large and irregular fluctuations characteristic of sleep. Under these conditions, the presentation of a bright projected area (see the signal on top) produces no effect; but in record 33C, in which the character of the base line is completely altered, visual stimulation causes a striking response. The difference between the "normal" waves of the two cats used in these experiments is probably related to the fact that the anesthetic used in the first preparation was pentothal, while in the second it was nembutal. In record 33C, the higher frequencies of the rhythm are absent during the stimulation period. They may have disappeared only because they were accelerated beyond the range of our instruments.

From a technical point of view, it is, of course, an essential observation that such a simple procedure can transform an apparently useless preparation into one from which records such as 33C may be taken. In our experiments, it soon became routine procedure to cause the arousal reaction whenever the symptoms of sleep appeared in the base line. But the observation that visual currents tend to disappear during sleep has also theoretical implications. If pattern vision as a psychological fact is directly related to the flow of visual currents, and if there is no vision in this sense during sleep, then the visual currents must disappear during sleep just as they do

under deep anesthesia. The direction of this argument cannot, of course, be reversed. The disappearance of visual currents during sleep and under deep anesthesia does not actually prove that these currents are directly related to vision in a psychological sense.

When a great many records had been taken, we could no longer doubt that, under conditions of steady stimulation, the visual currents of the cat have the surface-negative polarity. Thus we were brought back to the question whether visual currents recorded from intact human heads indicate a surface-positive polarity only because of technical complications inherent in this particular situation. So far no other cortical currents have been found which have the surface-positive polarity. A few years ago, Goldring and O'Leary ('51) studied the d.c. potentials of the rabbit's visual cortex when repetitive stimulation was applied either to the optic nerve or to the lateral geniculate nucleus. The experimental technique of the authors differed from the one used in our tests; moreover, they did not interpret their results as being related to visual perception. Nevertheless, these results are relevant to our problem. For, particularly when the optic nerve was stimulated, the recorded potentials had for the most part the surface-negative polarity.

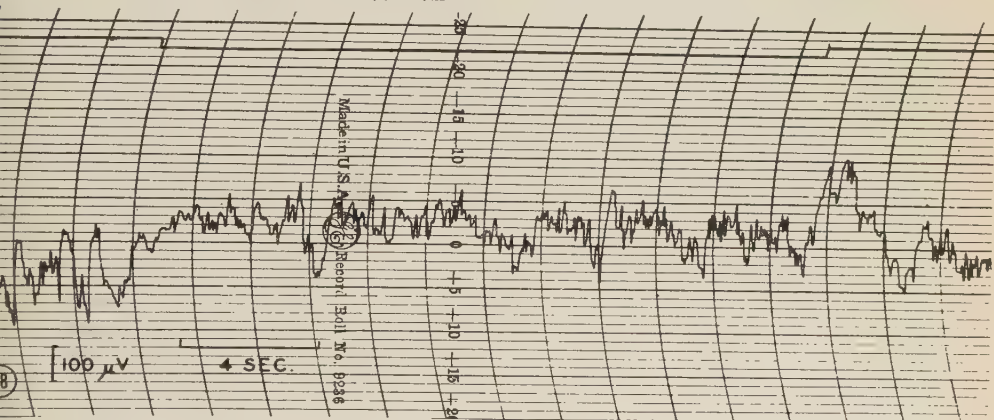
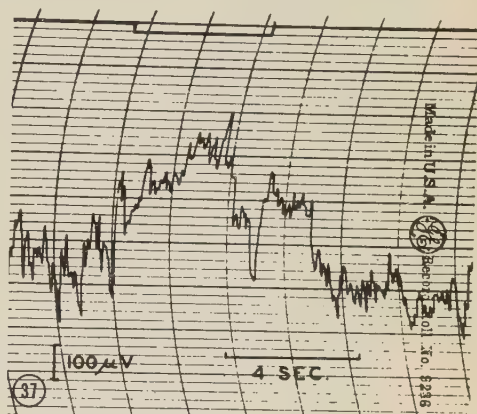
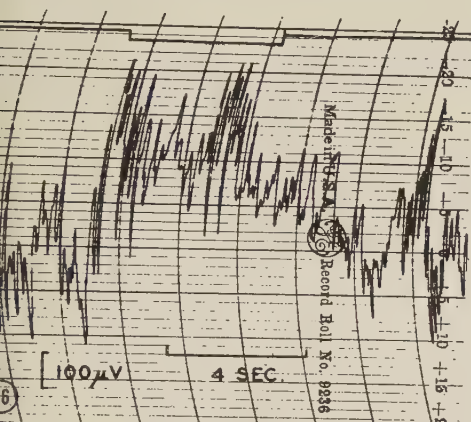
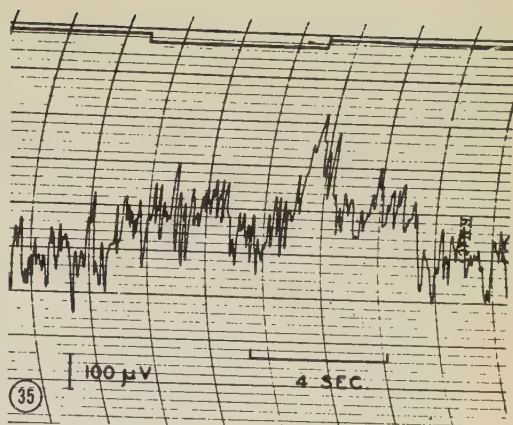
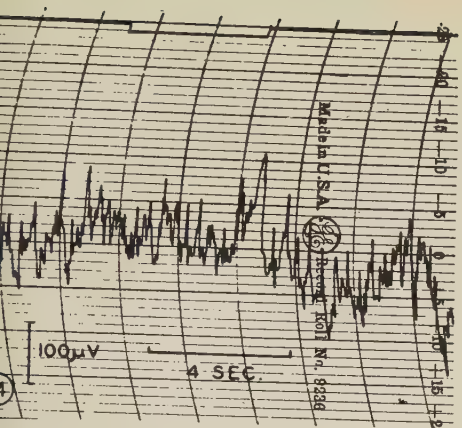
Sometimes, the cortical fovea of a human patient must be exposed for surgical purposes. If, in such a case, the wick of the active electrode could be placed on the dura of the exposed region, a few tests would probably suffice for answering the present question. Such experiments might prove that human visual currents also flow in the surface-negative direction. We have not yet found an opportunity to perform tests of this kind. In the meantime, it seemed to us desirable to obtain evidence as to the same problem from a further species, preferably from one more closely related to man. For this purpose, we recently took some records from the visual projection areas of three rhesus monkeys. No systematic investigation was intended or undertaken; but since, with regard to the question of

polarity, results were consistent, we feel justified in mentioning them briefly at this point. We are grateful for the kind help of Doctor Rosenblith, in whose laboratory at MIT the experiments were done, and of Doctor Pribram, who performed the necessary operations.

Parts of the occipital skull were removed under pentothal anesthesia. When the effect of the anesthetic had begun to subside, curare was applied in order to keep the animals quiet and, in particular, to prevent eye movements. Therefore, artificial respiration had to be used. The instruments and the electrodes were the same as in our work with cats. The active electrode made contact with the dura over exposed parts of the visual cortex, the grounded electrode with frontal bone, or the neck, or the dura over the inferior parietal region. In some instances, sudden bright illumination of the room was used as stimulation; in others, intense light was projected upon a white screen before the animal.

The records which we obtained seem to leave no doubt as to the polarity of the monkey's visual currents; just as those of the cat, these currents flow in the surface-negative direction (records 34 to 38). It does seem plausible to infer that human visual currents will show the same polarity when records are taken from the exposed cortical fovea. In this case, all now available records of such currents would have to be read upside-down.

Apart from the polarity shown in records 34 to 38, two points deserve to be mentioned. First, in record 38, the duration of stimulation amounts to almost 20 seconds. The response is not strong, but it persists until the end of the stimulation period. Secondly, not only in record 38 but also in the others there is intense cortical activity after stimulation. In records 34, 35, 37 and 38, this activity has the form of strong after-potentials; in 36, the continuation of the response after the stimulation period may also be caused by such a potential which, in this case, would merely have fused with the primary response. Never before have we seen after-potentials of this size in



records 34 to 38 Visual currents recorded from three rhesus monkeys (pentothal, curare, artificial iritation). Records 36 to 38 are taken from same animal. In record 38 the response persists for whole period of stimulation (19 sec.). All responses have the surface-negative polarity. Note usual size of after-potentials.

records of cortical currents. Further experiments will have to show whether curare has this curious effect. It might also be related to abnormal conditions which artificial respiration established in the tissue.

DISCUSSION

The present investigation has given new support to the thesis that activated parts of cortical projection areas are pervaded and surrounded by quasi-steady currents. The following remarks refer mainly to this general issue.

When we began our work on cortical currents, results appeared to be at odds with familiar physiological conceptions. We doubt whether this impression has since entirely disappeared. And yet, during the past twenty years, there have been other investigations to which our own studies seem to be closely related. Most probably, the connection is not always recognized. In the following remarks, we must therefore refer to these investigations even though they are, as such, well known.

Nobody can be disturbed by the thesis that activated histological elements are surrounded by fields which spread in the tissue as a volume conductor. This thesis is clearly based on facts, and is therefore accepted by all physiologists. The field of the nerve impulse has been measured at considerable distances from the active fiber. When the alpha rhythm of the human brain is registered from the intact head, it is, of course, only the field of the rhythm which reaches the crucial electrode. But the evoked potentials of cortical projection areas can also be recorded from intact human heads. Consequently, these potentials, too, have fields which spread freely around the activated cells. Under these circumstances, no further discussion of the concept of cortical fields seems necessary. (The present issue has been thoroughly analyzed by Adrian, '47, and by Bremer, '53.)

We will now turn to our finding that, in a state of *steady* excitation, activated parts of the cortex are sources of equally steady fields or currents. We see no reason why this finding

should be regarded as surprising. For, many years ago, steady potentials and corresponding currents have been demonstrated in other parts of the nervous system. At the time, neurophysiologists had become interested in certain non-propagated electric states of nervous tissue, namely, in those which impulses establish at synapses. The precise location of such states was not immediately clear. Later, the investigators' attention was mainly concentrated on non-propagated states which occur at post-synaptic cell boundaries. The following statements refer only to electric states which have been shown to be located at these boundaries. They are generally called synaptic (or post-synaptic) potentials. Synaptic potentials have been discovered in the ganglia of invertebrates, in the sympathetic ganglia of vertebrates, in the dorsal and ventral horns of their spinal cord, and at neuromuscular junctions. In some instances, specific chemical activities are involved in the origin of such potentials; but, in the present connection, we need not discuss this issue. It is not yet entirely clarified.

When one volley arrives at a synapse, a local potential is here established which then soon subsides. But if a further volley follows before this first effect has disappeared, an increased potential is often built up by summation. Many volleys of sufficient frequency tend to establish a steady electric state, although this state may still exhibit small fluctuations which correspond to the individual volleys.

The degree to which various synapses develop steady electric states is probably not the same in all cases. Quite apart from this fact, no entirely adequate records of such states can, of course, be taken when capacity-coupled amplifiers are used. Modern recordings do not often show that, under conditions of continued afferent action, synaptic potentials may persist for very long periods. Interest in these potentials as such is, however, growing fast (cf. Bishop, '56). It now remains to be seen whether the spreading of corresponding currents in the tissue as a volume conductor does not deserve equal attention.

Especially impressive records of steady synaptic states are shown in a well known article which Barron and Matthews

published in 1938. The potentials in question were those of synapses in the spinal cord, and the records clearly demonstrate how steady potentials are gradually established when volleys arrive at a growing rate. These potentials may then be maintained for large parts of a minute. Their voltage often amounts to several millivolt. Now, when potentials of this size arise in a cell layer, corresponding currents must immediately begin to flow through the activated parts and also through the surrounding tissue. It is now generally recognized that they do. In this sense, therefore, the records in question may be regarded as records of steady currents.

The synaptic currents of the spinal cord are particularly relevant to our own work because their sources are located in the central nervous system. It seems to us a plausible assumption that afferent impulses establish junctional potentials also at synapses in the cortical projection areas. If, under conditions of constant peripheral stimulation, such cortical synaptic potentials were as steady as the spinal potentials are under comparable circumstances, corresponding steady currents would, of course, flow through and around these cortical sources. Obviously, records of such currents would resemble those of steady spinal currents. But when our own records are compared with those which Barron and Matthews have taken from the spinal cord, no major difference will be found between these curves of different origin. At any rate, when registered under the right conditions, spinal synaptic potentials seem to be at least as steady as the potentials of cortical projection areas. We therefore suggest that the currents shown in our records are those of cortical synaptic potentials. If this is an acceptable thesis, the concept of cortical currents need no longer be regarded with suspicion. For, it now appears directly related to the generally accepted concept of synaptic currents in the spinal cord. In an earlier paper, we have mentioned this hypothesis with considerable reserve (Köhler and Wegener, '55). After a closer examination of the literature, we do not feel that so much caution is needed. It does seem natural to

assume that the steady currents of the cortex originate in the same way as the currents discovered in the spinal cord by Barron and Matthews.

Physiologists will mainly be interested in the consequences which follow from the present assumption. These consequences can tentatively be derived from the behavior of better-known synaptic currents. It will suffice if we mention only one point. When weak currents, the direction of which agrees with the polarity of synaptic potentials, pass through the motor cells of the spinal cord, they throw these cells and their fibers into rhythmic action. One would expect cortical synaptic currents to have the same effect. If this should prove to be true, cortical currents would play a major part in brain function. More specifically, Barron and Matthews have shown that the frequency of the efferent impulses which currents establish in a single cell of the ventral horn grows with the intensity of these currents. This would probably also be true of the impulses caused by cortical synaptic currents. But the frequency of the impulses aroused in an individual cell must be determined by the intensity of the flow which passes through this cell rather than by the intensity of the current as a whole. Consequently, the specific distribution of a cortical current as described in terms of local densities would, after translation into the language of frequencies, be represented in the efferent fibers. In this language, the pattern of the current at the source would now be transmitted to other parts of the brain or of the central nervous system in general.

SUMMARY

1. During stimulation by light quasi-steady currents can be registered from the visual cortex of the cat. These currents have the surface-negative polarity.

2. The temporal characteristics of such currents are approximately the same as those of the cat's auditory currents.

3. Under the conditions of our experiments, dark objects on a bright ground establish currents of the same polarity as bright objects on a dark background.

4. Responses to moving objects agree with expectations derived from known facts of cortical localization.

5. When the movement of an object is interrupted, the intensity of the visual current is rapidly reduced, presumably by increased anelectrotonic effects of the current itself.

6. Visual currents can be recorded when the eyes of the cat are immobilized.

7. The currents recorded from the visual projection area do not issue from the eyes.

8. No evidence has been found that, under the conditions of our experiments, light blocks the alpha rhythm of the cat's visual cortex.

9. There seem to be no visual currents during sleep. When the arousal reaction has occurred, the currents tend to appear immediately.

10. Some records of visual currents have been taken from the striate area of rhesus monkeys. These currents have the surface-negative polarity.

11. It is suggested that currents of the cortical projection areas are maintained by cortical synaptic potentials.

12. The distribution of current densities in activated parts of the cortex is likely to cause corresponding frequency distributions in the discharges of the neurons involved.

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